Cell proliferation and esophageal carcinogenesis in the zinc-deficient rat

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Target cell proliferation was investigated throughout the development of esophageal cancer induced by N-nitrosomethylbenzylamine (NMBA) in weaning rats maintained on zinc-deficient or sufficient diets. Deficient rats were fed ad libitum, while zinc-sufficient rats were either pair-fed to the deficient animals or fed ad libitum. After 5 weeks, half of the animals in each dietary group were given six intragastric doses of NMBA (2 mg/kg; twice weekly). The remaining rats were untreated by carcinogen. At weeks 1, 2, 3, 4, 5, 7, 9 and 11 post first dose, esophageal cell proliferation was assessed in rats from each group by in vivo bromodeoxyuridine (BrDU) labeling followed by immunohistochemical detection of cells in S-phase. At 11 weeks, the tumor incidence was 100, 23 and 6%, respectively, in the zinc-deficient, zinc-sufficient, ad libitum and pair-fed groups. In vivo BrDU labeling revealed that in the NMBA-untreated groups, the labeling index (LI), the number of labeled cells, and the total number of cells per cross section of entire esophagi were significantly increased by zinc deficiency at all time points; LI was lowest in zinc-sufficient, pair-fed rats. During NMBA treatment (weeks 6, 7 and 8), increased cell proliferation occurred in both groups of zinc-sufficient esophagi but only during week 6 in the deficient ones. In the weeks following the cessation of NMBA treatment, zinc-deficient esophagi showed significantly increased LI and greater number of labeled cells than the carcinogen treated, zinc-sufficient pair-fed or ad libitum fed groups. On the other hand, NMBA-treated zinc-sufficient pair-fed rats showed lower LI and smaller number of labeled cells than the zinc-sufficient ad libitum counterparts. Most importantly, esophageal papillomas were found in two zinc-deficient animals that had received no NMBA treatment, after 10–11 weeks of experimental diet. These data support a direct relationship between cell proliferation and tumor incidence, and also provide evidence that zinc deficiency and its associated cell proliferation could be carcinogenic.

Introduction

The first author and her colleagues (1) reported in 1978, that zinc deficiency increased the incidence and reduced the induction time of esophageal tumors in rats exposed to N-nitrosomethylbenzylamine (NMBA*). Similar findings were subsequently published by other workers (2–4) and extended to experiments in which the precursor amine and nitrite were simultaneously administered (5).

Rats maintained on a zinc-deficient diet develop proliferative changes in the esophagus including parakeratosis, hyperkeratosis and an increase in the number of cell layers (6,7). Schrager and his colleagues (8) noted that these earlier findings pointed to a relationship between zinc deficiency, cell replication and the incidence and growth of tumors. They investigated DNA synthesis and mitotic index in the esophageal epithelium during and after exposure of zinc-deficient animals to NMBA, and concluded that the enhancement of esophageal tumor incidence in the deficient animals was due, in part, to the increased proliferation of the target cells. At the time of publication of this work by Schrager et al. (8) there was already interest in the role of cell proliferation in chemical carcinogenesis and this has greatly increased in the intervening years (see Ref. 9 for list of references). However, opinion is divided between those emphasizing the importance of cell proliferation in carcinogenesis (10–12), and those expressing varying degree of skepticism (13,14).

The zinc deficiency-NMBA model in rats has many attractions for the further study of cell proliferation and chemical carcinogenesis because proliferation is induced by a reduced dietary intake of an essential trace metal and it can be reversed by its replenishment (Fong et al., unpublished data). Also, human epidemiological studies have implicated dietary zinc deficiency (15–17) and exposure to carcinogenic nitrosamines, and NMBA in particular, in the high incidence of esophageal cancer in Northern China and parts of Iran (16,18–20).

The work to be reported here confirms and extends that of Schrager et al. (8). By contrast with these authors, cell proliferation was measured by quantitative immunohistochemical visualization of 5-bromo-2′-deoxyuridine (BrDU, a thymidine analog) incorporation into the DNA of cells during DNA synthesis (S-phase) rather than [3H]thymidine incorporation into isolated DNA (8). Also, data were obtained throughout the experimental period up to the appearance of esophageal tumors, thus permitting quantitative comparison of rates of cell proliferation and tumor incidence in the animal. Continued observation of the group receiving the zinc-deficient diet alone revealed esophageal tumors in two animals against a background of prolonged cellular proliferation but no applied genotoxic injury.

Materials and methods

Animal diets

Custom-formulated, egg-white based zinc-deficient and control zinc-sufficient diets were prepared by Teklad (Madison, WI); they were identical except for the amount of zinc carbonate added. Zinc levels in diets were regularly monitored in our laboratory by atomic absorption spectroscopy, and were 4.3 ± 0.6 p.p.m. (22 samples) and 74.5 ± 4.9 p.p.m. (15 samples), respectively, for zinc-deficient and zinc-sufficient diets.
or left unstained for BrDU immunohistochemistry.

BrDU immunohistochemistry

Whole esophagi were excised, opened longitudinally, fixed in methacam for 3 weeks. T denotes time points at which five rats from each group were sacrificed following BrDU administration. Three consecutive doses of BrDU (10 mg/kg b.w.) were administered i.p. every 24 h and the animals were killed 2 h after the 3rd dose of BrDU. At the last time point all rats were killed for end point tumor incidence analysis following BrDU administration.

Experimental protocol

This study, approved by the Thomas Jefferson University Institutional Animal Care and Use Committee, was conducted under NIH guidelines. Weanling, 21-day-old, male Sprague-Dawley rats were purchased from Taconic Laboratory (Germantown, NY). The animals were maintained at 70 ± 2°F with a 12 h light-dark cycle. They were group-housed in suspended stainless steel cages and given access to deionized drinking water. The experimental design is depicted in Figure 1. Briefly, 330 animals were randomized into three dietary groups: Groups A and D were fed the zinc-deficient diet ad libitum; Groups B and E were pair-fed the control zinc-sufficient diet so as to match the food consumption of rats on the deficient diet, and Groups C and F were fed the zinc-sufficient diet ad libitum. All rats were weighed weekly. After 5 weeks on their respective diets, Groups D, E and F were given six intragastric doses of NMBA (Ash Stevens, Detroit, MI) over the course of 3 weeks. T denotes time points at which five rats from each group were sacrificed following BrDU administration. Three consecutive doses of BrDU (10 mg/kg b.w.) were administered i.p. every 24 h and the animals were killed 2 h after the 3rd dose of BrDU. At the last time point all rats were killed for end point tumor incidence analysis following BrDU administration so that esophageal cell proliferation was measured in all of these animals.

In vivo BrDU labeling

At each time point (Figure 1), animals from various groups were each administered three i.p. doses of BrDU, 10 mg/kg b.w., at 24 h intervals. The following injection timetable was adhered. BrDU administration was given between 7 and 10 a.m. on consecutive Wednesdays, Thursdays and Fridays, and the animals were killed 2 h after the third dose. During NMBA treatment (weeks 1, 2, 3, 4, 5, 7 and 9 post first dose of NMBA), esophageal cell proliferation was assessed in five rats taken randomly from each group by BrDU labeling followed by immunohistochemical detection of cells in S-phase. Groups A, B and C were without NMBA treatment; D, E and F were NMBA-treated. Groups A and D (−) were fed a zinc-deficient diet, ad libitum; C and F (−), a zinc-sufficient diet, ad libitum; B and E (+) pair-fed a zinc-sufficient diet. c denotes carcinogen treatment (NMBA, i.g., 2 mg/kg b.w., twice weekly for 3 weeks). T denotes time points at which five rats from each group were sacrificed following BrDU administration. Three consecutive doses of BrDU (10 mg/kg b.w.) were administered i.p. every 24 h and the animals were killed 2 h after the 3rd dose of BrDU. At the last time point all rats were killed for end point tumor incidence analysis following BrDU administration so that esophageal cell proliferation was measured in all of these animals.

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animals in all groups (data not shown). Serum samples from deficient rats (with or without NMBA treatment) had significantly lower levels of zinc at all time points, as compared to those in respective zinc-sufficient, ad libitum or pair-fed group (Table I). Similarly, zinc levels in testes of deficient rats, killed at the end point for tumor incidence analysis, were significantly lower than those in respective zinc-sufficient ad libitum or pair-fed group (Table I).

Table II shows that zinc deficiency significantly increased the incidence of esophageal tumors in rats given a cumulative dosage of 12 mg/kg NMBA over the course of 3 weeks. A 100% tumor incidence was seen in deficient rats versus 23 and 6%, respectively, in zinc-sufficient, ad libitum fed and pair-fed rats. Furthermore, tumor incidence was significantly lower in zinc-deficient, pair-fed rats than in zinc-sufficient ad libitum fed ones. The tumors were mostly exophytic papillomas with no apparent regional predilection, though, zinc-deficient rats appeared to have more and larger tumors in the upper one-third of the esophagus. In addition, tumor multiplicity was greater and tumor latency shorter in zinc-deficient rats. The number of tumors per esophagus was 8.70 ± 5.43, 0.43 ± 0.92, and 0.06 ± 0.02, respectively, in zinc-deficient, zinc-sufficient ad libitum fed, and pair-fed group, and tumors were first detected in two of the five deficient rats killed for cell proliferation measurement at 7 weeks post first dose of NMBA.

Most interestingly, esophageal polyps were found in two zinc-deficient rats that had not received NMBA treatment and, as expected, in none of the untreated zinc-sufficient pair-fed or ad libitum fed rats. The two zinc-deficient rats were killed for cell proliferation measurement at weeks 10 and 11. Each had three small polyps (about 0.5×1.5 mm) in the upper half of the esophagus and these were confirmed papillomas by histopathological examination (Figure 3). The significance of this observation (2/40 zinc-deficient had tumors versus 0/80 zinc-sufficient animals) was tested by the Fisher’s exact test. The two-tailed P-value is 0.1, which is not significant but suggestive.

### In vivo BrDU labeling methodology

Preliminary experiments were performed to determine (i) the dosage of BrDU, and (ii) the advantage of administering multiple doses versus a single dose of BrDU. Firstly, rats (five per group) were each given single i.p. injections of 10, 20, 50 or 75 mg/kg of BrDU, and killed 2 h later. No difference was found in their esophageal LI (%), which were 11.5 ± 1.2, 11.3 ± 1.8, 11.7 ± 1.1 and 12.6 ± 1.0, respectively. These data were in agreement with those of Schutte et al. (23), who found no increment in the LI of mouse small intestine between a BrDU dose range of 5–50 mg/kg. Secondly, rats were given two i.g. doses of NMBA at 2 mg/kg, or left untreated. At 24 h after the second NMBA dose, three consecutive doses of 10 mg/kg BrDU were administered i.p. at 24 h intervals. The esophageal LI (%) for NMBA-treated and untreated groups was 44.9 ± 10.8 and 27.0 ± 5.0, respectively. On the other hand, the corresponding LI for the NMBA-treated and untreated groups that received a single dose of BrDU at 30 mg/kg was 12.9 ± 4.2 and 11.5 ± 1.8, respectively. These data demonstrate that repeated pulse injections of BrDU increased the sensitivity of LI measurement of chemically-induced cell proliferation. In this respect, Eldridge et al. (24) reported that BrDU administered in a 3-day osmotic pump was the method of choice for assessing chemically-induced cell proliferation. In view of the large number of animals involved in the present study, we decided to achieve continuous labeling (25) by giving three repeated i.p. pulse injections of BrDU at 10 mg/kg every 24 h over the course of 3 days.

### Zinc deficiency, food restriction and esophageal cell proliferation

Histopathologically, there was no apparent difference between an esophagus from a rat fed a zinc-sufficient diet ad libitum and those in respective zinc-sufficient, ad libitum or pair-fed groups (Table I). Similarly, zinc levels in testes of deficient rats, killed at the end point for tumor incidence analysis, were significantly lower than those in respective zinc-sufficient ad libitum or pair-fed group (Table I).

| Table I. Serum and testis zinc levels in zinc-deficient and zinc-sufficient rats. Results are means ± SD on a sample size of 27–60 rats per group as indicated by the number in parenthesis. Serum samples from NMBA-untreated and NMBA-treated rats were from individual rats killed at various time points indicated in Figure 1. Testis samples were from animals killed at the conclusion of the tumorigenesis study. |
|---|---|---|
| Group | Zinc levels (µg/100 ml) | Testis (p.p.m.) |
| NMBA-untreated | | |
| Zinc-sufficient, pair-fed | 133 ± 18 (30) | – |
| Zinc-sufficient, ad libitum fed | 145 ± 25 (34) | – |
| Zinc-deficient, ad libitum fed | 56 ± 7* (27) | – |
| NMBA-treated | | |
| Zinc-sufficient, pair-fed | 143 ± 29 (60) | 209 ± 16 (35) |
| Zinc-sufficient, ad libitum fed | 152 ± 23 (65) | 198 ± 8 (29) |
| Zinc-deficient, ad libitum fed | 59 ± 12b (60) | 141 ± 24* (35) |

*Significantly different from respective zinc-sufficient, ad libitum and zinc-sufficient pair-fed groups (a and b, P < 0.001; c, P < 0.02), using Student’s t-test and Bonferroni correction was performed on the data. Difference was not significant between zinc-sufficient ad libitum groups and pair-fed groups.

| Table II. Incidence of esophageal tumors induced by low doses of NMBA in male Sprague–Dawley rats fed zinc-deficient and zinc-sufficient diets. The rats were fed their respective diets for 5 weeks. NMBA was then administered i.g. at a dose of 2 mg/kg b.w., twice weekly for 3 weeks. All animals were killed 11 weeks post first dose of NMBA. Number in parentheses are percentage of tumor incidence. The zinc content of the deficient and sufficient diets were 4 and 75 p.p.m. respectively. |
|---|---|---|---|---|---|
| Group | Diet | Cumulative NMBA dose mg/kg | Tumor incidence | No. of tumors/esophagus | Esophaghi with tumors of size > 2×2 mm |
| | | | | 1 | 2 | 3 | 4-6 | >7 |
| D | Zinc-deficient, ad libitum | 12 | 40/40 (100)* | 0/40 | 6/40 | 6/40 | 8/40 | 20/40 | 26/40 |
| E | Zinc-sufficient, pair-fed | 12 | 2/35 (6)* | 2/35 | 0/35 | 0/35 | 0/35 | 0/35 | 0/35 |
| F | Zinc-sufficient, ad libitum | 12 | 7/50 (23) | 4/30 | 0/30 | 3/30 | 0/30 | 0/30 | 0/30 |

*Significantly different from respective zinc-sufficient, pair-fed and zinc-sufficient, ad libitum fed groups; P < 0.001, using chi-square analysis.

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Fig. 3. Pedunculated esophageal squamous cell papilloma projecting into esophageal lumen from a rat fed a zinc deficient diet for 10 weeks, in the absence of NMBA treatment. Counterstaining with H&E. Microscope setting ×100.

and that from one pair-fed the same diet. Typically, the esophagus from either group showed a single layer of basal cells, with an overlying stratum two to three cells thick covered by a thin keratinous layer (Figure 4a and b). By contrast, the esophageal basal layer from a zinc-deficient rat after 9 weeks of deficient diet was markedly thickened and dysplastic. The overlying stratum was increased in width, averaging 6 to 10 cells in thickness, and the keratinous layer showed parakeratosis and hyperkeratosis (Figure 4c).

In vivo BrDU labeling and immunohistochemistry revealed significant differences in the pattern of esophageal cell proliferation between the three groups of carcinogen-untreated animals (Figure 5a, b and c). Invariably, zinc-sufficient, pair-fed rats had the lowest number of labeled cells (Figure 5a) and zinc-deficient rats the highest number (Figure 5c). Furthermore, the distribution of BrDU-labeling was quite even along the entire length of the esophageal epithelia of both groups of zinc-sufficient rats (Figure 5a and b), although the zinc-deficient rat esophagus often displayed higher levels of labeling in hyperplastic areas (Figure 5c). Higher levels of labeling were also often associated with tumors, and dysplastic areas in NMBA-treated rats (results not shown).

Quantitatively, zinc-deficient rats showed significantly increased esophageal LI as compared to respective zinc-sufficient, pair-fed, or ad libitum fed groups (Figure 6). Furthermore, the zincufficient, pair-fed group displayed the lowest LI at all time points, and the zinc-deficient group the highest. The LI (%) ranged from 10.7 ± 2.1 to 17.0 ± 3.1, 27.0 ± 3.0 to 33.4 ± 3.7 and 38.4 ± 4.7 to 50.5 ± 5.9, for zinc-sufficient pair-fed, ad libitum fed, and zinc-deficient groups, respectively. There was also a significant difference in LI between the zinc-sufficient pair-fed and respective ad libitum fed group at all time points. Figure 7 shows that zinc-deficient rats had significantly more labeled cells per cross section of an entire esophagus than either their respective zinc-sufficient, pair-fed group at all time points, or zinc-sufficient, ad libitum group at most time points. In addition, zinc-sufficient, pair-fed rats had significantly fewer labeled cells in esophageal epithelia than their respective zinc-sufficient, ad libitum group at most time points.

Lastly, there was no significant difference in the total number of cells (labeled plus unlabeled, range: 1162 ± 171 to 1633 ± 269 per cross section of an esophagus) between the two groups of zinc-sufficient rats. On the other hand, the total number of cells in deficient esophagi (range: 1615 ± 201 to 3497 ± 626) was significantly higher than those in the two zinc-sufficient groups at several time points (Figure 7). These data support the observation by earlier investigators that zinc-deficiency induced hyperplasia in rat esophagus (6,7).

Cell proliferation and NMBA-treatment

During carcinogen dosing, significantly increased LI and enhanced number of labeled cells occurred in both groups of
Zinc deficiency, cell proliferation and esophageal carcinogenesis

Fig. 5. Microphotographs showing typical pattern of immunostaining with BrDU monoclonal antibody to detect cells in S-phase in esophagus from rats maintained on the following dietary regimen for 9 weeks: (a) pair-fed a zinc-sufficient diet; (b) ad libitum fed a zinc-sufficient diet; and (c) ad libitum fed a zinc-deficient diet. Counterstaining with hematoxylin. Microscope setting x200.

zinc-sufficient esophagi (Figures 6 and 7). These levels did not persist and returned to the level of those in the respective untreated group within 10 days after the 6th dose (week 9). On the other hand, zinc-deficient rats only showed increased cell proliferation during the first week of NMBA treatment as compared to the respective untreated zinc-deficient group or NMBA-treated zinc-sufficient groups. Following the cessation of NMBA treatment, zinc-deficient rats showed more labeled cells than the respective zinc-sufficient, pair-fed group (weeks 9, 10, 12, 14 and 16, Figure 7), and zinc-sufficient ad libitum fed group (weeks 12, 14 and 16, Figure 7), but also had higher LI than the corresponding pair-fed group (weeks 9, 10, 12, 14 and 16, Figure 6), and ad libitum group (weeks 10, 12, 14 and 16, Figure 6). In this respect, after NMBA treatment was stopped, zinc-sufficient, pair-fed rats showed significantly lower numbers of labeled cells (weeks 9, 10, 12, 14 and 16, Figure 7), and also lower LI (weeks 9, 10, 12, 14 and 16, Figure 6) than the respective ad libitum fed rats.

Discussion

Our data support the hypothesis that increased and sustained cell proliferation is associated with an increased tumor risk.
after exposure to a chemical carcinogen (10–12,26) and its corollary that reduced proliferation improves the odds of this risk. They also show that zinc deficiency alone, with its associated increased cell proliferation, can be carcinogenic in the rat esophagus.

The increased cell proliferation produced by zinc deficiency is specific since food restriction in the zinc sufficient pair-fed rats did not bring about an increase but rather a reduction in esophageal cell proliferation. In this respect, Schrager et al. (8) reported that dietary zinc deficiency signiﬁcantly increased the incorporation of [3H]thymidine into DNA isolated from esophageal epithelium measured at 14 and 28 days after the rats were fed a deﬁcient diet, but pair feeding signiﬁcantly decreased incorporation into the esophageal DNA relative to the ad libitum diet at 14 days but not at 28 days. By contrast, our results show that both the increased, and reduced cell proliferation induced by dietary zinc deﬁciency, and pair feeding respectively, were sustained over the entire experimental period of 11 weeks. Several studies using the technique of in vivo [3H]thymidine labeling have also shown that calorie restriction leads to a reduction in labeling indices in esophagus and other tissues of the mouse (27,28) and colon of the rat (29).

During NMBA treatment, the increased esophageal cell proliferation that occurred in both groups of zinc-sufficient rats did not persist, but returned to the level of that in the respective NMBA-untreated groups after the cessation of carcinogen treatment (Figures 6 and 7). In support, histological examination of esophageal sections revealed that as early as 10 days after the completion of NMBA dosing, regeneration of basal cells began, though with considerable variation between rats in the extent of this process (results not shown). This is probably due to the large difference in the severity of these lesions in the ﬁrst place. These observations were consistent with those of Craddock and Driver (30), who showed that repeated injection of 2 mg/kg NMBA to rats resulted in basal cell necrosis and a massive inﬂux of inﬂammatory cells, followed by a reduction in severity in both events even in the presence of the carcinogen and, ﬁnally, by regeneration of the basal epithelial layer. Furthermore, our data are in agreement with those of Kokkinakis and Subbarao (31), who reported that the increase in DNA synthesis during carcinogen treatment in the hamster pancreatic cancer model did not persist. These authors attributed the increase to the greater number of cells entering S-phase, rather than to an increase in the rate of proliferation of a certain population of cells.

In the already high level of cellular proliferation induced by dietary zinc deﬁciency, further stimulus by low doses of NMBA did not elicit an increase in esophageal cell proliferation in zinc-deﬁcient rats during dosing as it did in the zinc-sufﬁcient groups (Figure 6). It is likely that the effect of NMBA on cell proliferation was masked by that induced by zinc deﬁciency. However, NMBA-treated zinc-deﬁcient rats displayed higher LI and a larger number of labeled cells than the respective untreated, zinc-sufﬁcient NMBA-treated group during dosing as it did in the zinc-sufﬁcient NMBA-treated group. The likely explanation of why NMBA did not elicit the expected increase in proliferation in the zinc-deﬁcient rats in our study and in that of Schrager et al. (8) is that the two effects (NMBA and zinc deﬁciency) were additive, not synergistic. The results of our study suggest that the effect of NMBA on cell proliferation in the esophagus is limited to those cells that are already proliferating at an abnormally high rate and that low-dose NMBA, which is effective in the ductal exocrine pancreas, is ineffective in the esophagus.
assessed DNA synthesis by $[^{3}H]$thymidine incorporation into isolated DNA of esophageal epithelia with the DNA label given 90 min before sacrifice.

Recently, Siglin et al. (32) evaluated NMBA tumorigenicity in rats following various short-term subcutaneous treatment regimens and reported significant difference in esophageal LI between control and NMBA-treated rats at end point tumor analysis. A possible explanation for the discrepancy between their study and ours is that different criteria were used in LI determination. We measured LI by scoring BrDU-labeled cells in S-phase whereas Siglin et al. (32) counted all PCNA (proliferating cell nuclear antigen)-labeled cells. Since the synthesis of PCNA, an endogenous marker of cell proliferation, appears to begin in G1 and to peak during the S-phase of the cell cycle (33,34), positive staining for PCNA includes not only cells in S-phase but also those in G1/S phase, G2-phase and during mitosis (35,36). These authors (32) also reported a higher LI for carcinogen-untreated controls than our data for zinc-sufficient ad libitum fed ones.

Thus, in this rat model in which sustained cell proliferation became the major variable under study (14), we have shown an association between cell proliferation and tumor incidence. By 11 weeks, zinc-deficient rats showing the most esophageal cell proliferation throughout tumor development had the highest (100%) tumor incidence (Table II). Zinc-sufficient pair-fed rats that demonstrated reduced esophageal cell proliferation relative to the ad libitum fed group had the lowest tumor incidence (Table II). The latter results are in line with other workers' reports that calorie restriction decreased the incidence of carcinogen-induced colon and mammary cancers in rats (37-39).

The important role of sustained and increased cellular proliferation in esophageal carcinogenesis is further borne out by our observation that esophageal papillomas were detected in two zinc-deficient rats in the absence of genotoxic injury by NMBA after 10 and 11 weeks of zinc deficient diet, respectively. These results thus suggest an answer to the important question whether the stimulation of cell proliferation alone can lead to a carcinogenic response (9). There are several possible explanations for this to occur: (a) Cellular DNA does not replicate with 100% fidelity (40), and cell proliferation provides a greater opportunity for spontaneous genetic errors to occur, thereby increasing the risk of developing cancer (11); (b) enhanced cell proliferation is possibly brought about by perturbation of cell-cycle controls, which could increase spontaneous DNA damage and lead to tumor initiation (9); (c) increased cell proliferation decreases the time that is available for DNA repair and thus fixes the errors (41), and may increase the expression of certain oncogenes that are otherwise expressed only weakly (42); or (d) increased rate of DNA repair is possible in zinc deficiency and is likely to be error prone (43).

NMBA undergoes P450 isozyme bioactivation to produce benzaldehyde and an electrophilic methylating agent (44), which methylates DNA, resulting in the formation of the promutagenic adduct, $O^{6}$-methylguanine ($O^{6}$-meG) and $N^{7}$-methylguanine (45-47). The enhanced carcinogenic activity of NMBA observed in zinc deficiency, is probably brought about by a combination of (a) increased esophageal microsomal metabolism of NMBA (4); (b) increased formation of the promutagenic adduct, $O^{6}$-meG in the esophagus (48); (c) depressed esophageal activities of the repair enzyme for $O^{6}$-meG, namely, $O^{6}$-alkylguanine-DNA-methyltransferase (49); and (d) increased esophageal cell proliferation, as demonstrated in this study. The present data suggest that this cell proliferation is likely to be the rate-determining factor of tumor incidence after low doses of NMBA. Further experiments are required to elucidate this point.

Lastly, in human studies, increased esophageal cell proliferation measured by in vitro $[^{3}H]$thymidine labeling of cells in S-phase was reported among residents in a high risk group for esophageal cancer in China (50,51). Furthermore, an increase in the overall LI was found as the normal esophageal epithelium progressed to hyperplasia, mild dysplasia and finally to dysplasia (52). These findings support the hypothesis that an increased risk of cancer is linked to increased esophageal cell proliferation (52). Nutrition intervention studies in high risk areas for esophageal cancer in China reported that supplementation with calcium had no effect on the observed abnormal esophageal cell proliferation patterns (53). Calcium supplementation also had no inhibitory effect on NMBA-induced esophageal carcinogenesis in rats (54). On the other hand, supplementation with multiple vitamins and minerals including 45 mg of zinc daily for 30 months resulted in lower values in the measure of the vertical distribution of labeled cells in the esophageal epithelium but had no effect on the overall labeling index (55). Thus, the zinc deficient-NMBA rat model has human relevance and provides opportunities to investigate the role and mechanism of cell proliferation in esophageal carcinogenesis.

In summary, we have provided quantitative data to: (a) indicate the importance of sustained enhanced and reduced cell proliferation in esophageal carcinogenesis; (b) support a direct relationship between cell proliferation and tumor incidence; and (c) show that zinc deficiency alone with its associated increased cell proliferation can be tumorigenic in the rat esophagus. These results support the view that abnormal cellular proliferation must be taken into account in the evaluation of bioassays of environmental chemicals and emphasize the importance of attempts to control it by chemopreventive treatment regimes.

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