Induction of Cell Proliferation in the Forestomach of F344 Rats Following Subchronic Administration of Styrene 7,8-Oxide and Butylated Hydroxyanisole

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ABSTRACT

The question addressed was whether stimulation of cell proliferation could be responsible for tumor induction in the forestomach by styrene 7,8-oxide (SO). Male F344 rats were treated for 4 weeks with 0, 137, 275, and 550 mg/kg SO by p.o. gavage 3 times/week. Positive controls received 0, 0.5, 1, and 2% butylated hydroxyanisole (BHA) in the diet for 4 weeks. Twenty-four h before termination of the experiment, the rats were implanted s.c. with an osmotic minipump delivering 5-bromo-2'-deoxyuridine (BrdU). Cell proliferation in the forestomach was assessed by immunohistochemistry for BrdU incorporated into DNA. Cell number/mm section length and fraction of replicating cells (labeling index) were determined in 3 domains of the forestomach, the saccus caecus, the midregion, and the prefundic region. With the exception of the prefundic region of the low-dose SO group, a significant increase of the labeling index was found in all regions both with SO and BHA. Rats treated with BHA showed, in addition, a dose-dependent increase in number and size of hyperplastic lesions. This was most pronounced in the prefundic region where carcinomas were reported to be localized. In this region, the number of dividing cells/mm section length was increased up to 17-fold. With SO, only marginal morphological changes were occasionally observed, despite the fact that the respective long-term treatment had been reported to result in a higher carcinoma incidence than treatment with BHA. It is concluded that the rate of replicating cells alone, numerically expressed by the labeling index, is an insufficient tool for interpreting the role of cell division in carcinogenesis. It is postulated that SO and BHA induce forestomach tumors via different mechanisms. While hyperplasia in the prefundic region most likely dominates the carcinogenicity of BHA, a mechanism combining marginal genotoxicity with strong promotion by increased cell proliferation appears to be involved in the tumorigenic action of SO.

INTRODUCTION

Research in chemical carcinogenesis is increasingly confronted with situations where the stimulation of cell division is believed to be the main risk factor (1–3), e.g., by accelerating the fixation of DNA lesions as mutations (4, 5) or by increasing the loss of heterozygosity of tumor suppressor genes by mitotic recombination (6–8). Stimulation of cell division can be the result of (high-dose) cytotoxicity and regeneration or of a hormone-like mitogenic activity.

In bioassays for carcinogenicity, both SO[2] (the most important intermediate metabolite of the plastic monomer styrene) and BHA (an antioxidant used in foods) induced squamous cell carcinomas in the forestomach of either sex of F344 rats (9–12). At maximum tolerated doses, the incidence reported in male rats was 84% for SO [p.o. gavage of 550 mg/kg, 3 times/week (11)] and 22% for BHA [2% in the diet (12)].

SO was genotoxic in several in vitro tests (13), but DNA binding in the forestomach was below the limit of detection (14). Tests for genotoxicity with BHA were generally negative (15–17). Both SO and BHA were clearly cytotoxic in vitro (18, 19) and were shown to bind

to protein in vivo (20, 21). It is possible, therefore, that SO and BHA were carcinogenic in the forestomach via a similar "nongenotoxic" mechanism of action, i.e., the stimulation of cell division induced by cytotoxicity and regenerative hyperplasia. This mechanism has been postulated for various forestomach carcinogens (22). BHA was demonstrated to have a strong hyperplasiogenic activity (17, 23), particularly in the prefundic region of the forestomach, i.e., close to the ridge forming the limit to the glandular stomach. This was also the region where BHA-induced squamous cell carcinomas were predominantly localized, a correspondence that might be of value for the analysis of the mechanism of carcinogenic action.

In this study, SO was examined for an effect on cell proliferation kinetics in the forestomach. The activity of SO was compared to the one seen with the "nongenotoxic" forestomach carcinogen BHA, in order to investigate whether tumor induction by SO could be explained by a similar mechanism of action.

MATERIALS AND METHODS

Application Solutions and Food

Solutions of SO (purity >98%; Merck, Darmstadt, Germany) in corn oil (Kentaur-Nuxo, Burgdorf, Switzerland) were prepared biweekly, in accordance with the conditions described in the 2-year bioassay (11). For the highest dose, for instance, 5.2 ml SO were mixed with 4.8 ml corn oil.

BHA (>98% 3-t-butyl-4-hydroxyanisole) was from Fluka (Buchs, Switzerland), powdered lab chow (No. 890) from Nafag (Gossau, Switzerland). The appropriate amounts of BHA (e.g., 60 g BHA for the 2% diet) were dissolved in 100 ml diethyl ether and poured onto 400 g powdered food. A homogeneous slurry was prepared, the ether was evaporated under ambient air (60 h), and the premix was pulverized in a mortar and mixed for 0.5 h in a food-mixing machine into powdered food to make up for a total of 3 kg food (e.g., 2540 g for the 2% diet).

Animals and Treatments

Forty 7-week-old male F344 rats from Charles River Wiga (Sulzfeld, Germany) were randomized by weight into groups of 5 animals. They were held in Macrolone cages on hardwood chip bedding and were acclimatized for 12 days with free access to tap water and maintenance diet 890 (Nafag). The SO animals received pelleted diet all the time, whereas the BHA animals were switched to powdered food after 1 week of acclimatization.

During the 4-week treatment period, the SO-rats were exposed to 0, 137, 275, and 550 mg/kg SO by p.o. gavage in corn oil (1 ml solution/kg body weight), 3 times/week (Mondays, Wednesdays, and Fridays at 8:00 a.m.). The BHA concentration in the powdered food was 0, 0.5, 1, and 2%. Consumption of BHA-containing food and body weights were measured 3 times/week. Animals were observed daily for signs of toxicity and behavioral changes.

Cell Proliferation Studies

Implantation of Minipumps. Alzet osmotic minipumps (Model 2001D, lot 047001; flow rate, 9.1 μl/h; 1-day delivery; Alza Corp., Palo Alto, CA) were filled with 220 μl BrdU (lot 21H0066; Sigma, St. Louis, MO) solution (20 mg BrdU/ml; 10 mM potassium phosphate; 130 mM NaCl; pH 7.6 (PBS); 1% 1 N NaOH) and implanted s.c. in the upper back of the rats on Monday of week 5, in the afternoon after a final SO treatment.

Histopathology and Immunohistochemistry. After 24 h of single caging, the animals were killed, the stomach and a piece of the upper small intestine were removed, and the stomach was cut open along the minor curvature, rinsed

in 0.9% aqueous sodium chloride, pinned flat on a cork board, and fixed in buffered formalin for 1 week. A 3-mm thick strip was cut along the major curvature, embedded in Paraplast paraffin (Monoject Scientific Inc., Athy, Ireland), and cut into 2-μm thin sections.

The sections were stained immunohistochemically for the incorporation of BrdU into DNA (24). This procedure involved using a monoclonal primary antibody (anti-BrdU murine IgG; Becton Dickinson Co., Research Triangle Park, NC) which was detected with a secondary link antibody (biotinylated goat anti-mouse IgG), linking the primary antibody to a label (streptavidin) coupled with alkaline phosphatase. Fast red was used as a chromogen. Link and label were components of a Supersensitive Kit purchased from BioGenex Laboratories, Inc. (San Ramon, CA). The sections were counterstained with hematoxylin.

Assessment of Cell Numbers and Labeling Index. BrdU-positive cells had red nuclei, and the negative nuclei stained blue. Cells were counted in 3 defined anatomical regions of the forestomach, the saccus caecus (near the esophagus), the midregion, and the prefundic region (near the limiting ridge to the glandular stomach). Only cells in the cohesive cell layers were counted. The average number counted in a 1-mm section length was 220. In the hyperplastic regions, cells were counted only along 0.5- or 0.25-mm section length, which resulted in 350–900 cells. Results are given (a) as number of dividing cells/mm section length and (b) LI as the ratio of the number of dividing cells/mm section length over the total number of cells/mm section length. One section was evaluated per rat. An average was calculated for the groups of 5 rats and expressed as mean ± 1 SD. Differences between the groups were analyzed with a 2-sided Fisher test.

RESULTS

Food Consumption and Body Weight

The animals administered BHA in the diet initially showed a dose-dependent decrease in food intake. After 4–5 days of treatment, no difference was seen between the groups. On a 4-week average, rats receiving BHA in the diet consumed 70–71 g food/kg/day, respectively. The BHA average dose consumed was calculated to be 350, 710, and 1400 mg/kg/day for the 3 groups (0.5, 1, and 2% BHA in the diet). The mean body weight of the animals receiving 1 and 2% BHA in the diet decreased in the first week of treatment but recovered as soon as the animals had adjusted to the BHA-supplemented food. The animals receiving SO 3 times/week by gastric intubation did not show any difference in food intake, weight gain, or behavior when compared to controls.

Gross Pathology

In the animals treated with 1 and 2% BHA, gross examination of the forestomachs revealed some alterations in the prefundic region, specifically near the limiting ridge. These changes showed up as a thick white but flimsy layer covering the forestomach mucosa. The forestomach mucosa also appeared thicker in treated animals when compared to that of the controls. No gross pathological alterations were observed in control animals or in the animals treated with SO and in the corresponding vehicle controls.

Histopathology

SO Groups and Controls. No pathological changes were observed in the forestomachs of control animals given corn oil by p.o. gavage 3 times/week, with the exception of 1 animal which showed a very mild hyperplasia and hyperkeratinization in the saccus caecus and the midregion of the forestomach. A marginal increase in the thickness of the squamous epithelium and a slightly enhanced keratinization of the forestomach was observed in the SO-treated rats (Fig. 1A). Two animals, one of each of the intermediate- and high-dose groups, showed a mild hyperplasia in the midregion.

BHA Groups and Controls

No pathological alterations were observed in any of the control animals fed with the standard diet in powdered form. On the other hand, a dose-dependent increase in number and severity of histopathological lesions became apparent when inspecting the forestomachs of the BHA-treated animals.

Rats treated with 0.5% BHA in the diet showed mild hyperkeratinization and an increased thickness of the squamous epithelial layer. One animal had a mildly hyperplastic lesion near the limiting ridge. This hyperplasia was characterized by hyper- and parakeratosis, dyskeratosis, some loss of polarity of the stratified cell layer, and slight nuclear polymorphism. However, no papilloma-like projections were observed and the lesions were clearly exophytic.

All animals treated with 1% BHA showed squamous epithelial hyperplasia and hyperkeratosis near the limiting ridge, while few animals revealed hyperplastic lesions in the midregion of the forestomach. No lesions were observed in the saccus caecus.
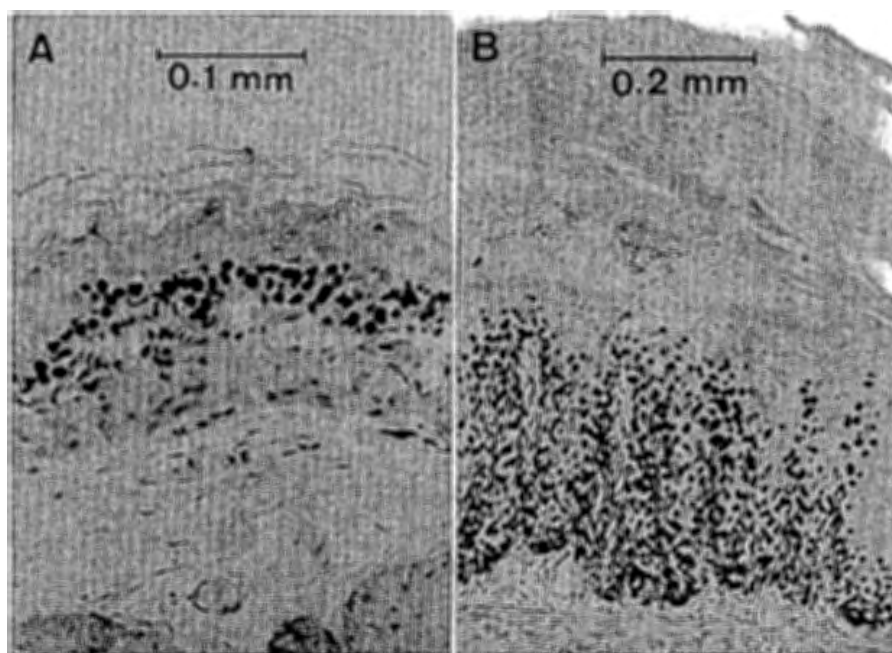

Fig. 1. Histopathological observations in the forestomach of male F344 rats following treatment with 550 mg/kg styrene oxide by gavage 3 times/week for 4 weeks (slight hyperkeratinization) (A) and 2% BHA in the diet for 4 weeks (severe diffuse hyperplastic lesion) (B).

In the group treated with 2% BHA, varying degrees of hyperplasia were seen in all rats (Fig. 1B) and, in one case, possibly a papilloma near the limiting ridge. In 4 out of 5 animals, the hyperplasia was accompanied by a few small dysplastic nodules and mild nuclear polymorphism. In three animals, mild hyperplastic lesions were found in the midregion of the forestomach. Hyperkeratinization was evident in all three anatomical regions of the forestomach, although it was most extensive near the limiting ridge and in the midregion. Despite the severity of some of the lesions, all lesions were exophytic.

Cell Proliferation

Labeling Index. The 4-week exposure of rats to both SO and BHA increased the rate of proliferation in all regions of the forestomach (Fig. 2).

With SO, in the saccus caecus, the LI increased from 49.1 (control group) to 70.3% (550 mg/kg SO), in the midregion from 42.6 to 59.2%, and in the prefundic region from 41.6 to 54.3%. No clear dose-response relationship was seen. Within the same dose group, including corn oil controls, the LI tended to decrease from the saccus caecus to the prefundic region. Since this is not seen in the control group of the BHA group, it may be a vehicle (corn oil) effect.

BHA also increased the rate of cell division in all treated groups. In the saccus caecus, the LI was raised from 37.3 (control group) to 50.6% (2% BHA), in the midregion from 42.7 to 59.7%, and in the prefundic region from 41.3 to 55%. Again, no dose response was seen, nor were there any regional differences.

In contrast to the pathological findings, the effects of SO and BHA on the labeling index were indistinguishable both with respect to anatomical region and level of induction. The lack of a clear dose-response relationship indicates that the cell proliferation response had reached its biological maximum when measured with a 24-h integral for the number of cells in S phase.

Number of Cells "at Risk". Because the LI did not indicate regional differences, yet both gross and histopathological observations as well as tumor localization showed regional differences after BHA treatment, the total number of cells/mm section length and the number of dividing cells/mm section length were determined. For the latter, the results are shown in Fig. 3. In the rats given SO, the number of dividing cells/mm section length increased merely in proportion to the increase of the LI, at the most by a factor of 1.6. The effect was similar in all forestomach localizations, in agreement with the lack of a site specificity of the (minor) histopathological findings.

Exposure to dietary BHA, on the other hand, caused a dramatic increase, especially in the prefundic region (Fig. 3). At 0.5%, 2 of 5 animals showed a 3-fold increase in the number of dividing cells/mm in this particular region of the forestomach. Due to the considerable increase in the animal-to-animal variability, the statistical significance
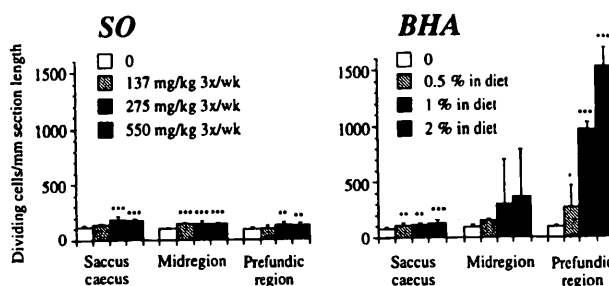

Fig. 3. Number of dividing cells/mm section length in three regions of the forestomach of male F344 rats following p.o. gavage of SO 3 times/week for 4 weeks, and BHA at 0–2% in the diet for 4 weeks. Mean values ± 1 SD. *, $P < 0.05$; **, $P < 0.025$; ***, $P < 0.0025$; 2-sided Fisher test.

of the effect was not very high. With 1% BHA, all animals showed a marked increase in the number of dividing cells in the prefundic region. In the 2% group, this effect was even more dramatic: a 17-fold increase in the number of dividing cells/mm section length was found. The region of the saccus caecus showed only effects related to the increase in the LI, similar to the situation with SO. In conclusion, cell numbers described the reaction of the squamous epithelium much better than the LI, both with respect to histological changes after 4 weeks and with the localization of the forestomach tumors after 2 years.

DISCUSSION

Four-week exposure of rats to the two forestomach carcinogens SO and BHA resulted in a significant increase in the rate of cell proliferation in the forestomach, as expressed by the LI. The LI in the controls was at 40%. This is high in comparison with another report (25) which was based on Sprague-Dawley rats. Differences to published LIs can also be due to the age of the animals (26), to the fact that only the basal cell layers were considered, and to the use of 1-day osmotic minipumps. Under the latter conditions, all cells in S phase anytime within a period of 24 h will be labeled. The advantage of this method in comparison with pulse labeling by BrdU injection is that diurnal cycling of DNA synthesis is integrated so that cycle differences between individual animals are evened out. The disadvantage is that high LI in the controls reduce the range available for detecting an increase. In this study, significant effects could be observed, indicating that the minipump method was appropriate.

Stimulation of cell division is a widely accepted indicator of non-genotoxic carcinogenesis in the forestomach (22). Our data show that information on the LI alone is not sufficient to characterize carcinogenesis in the forestomach by a nongenotoxic carcinogen. When comparing the effects induced by SO and BHA, the increase in the LIs was similar despite a number of clear differences in tumor incidence and tumor localization. The LI did not either reflect the marked morphological changes observed after 4 weeks of BHA treatment. Hence, an increased LI only indicates that a higher percentage of a certain cell population is dividing but it does not take into account the size of the populations. Much more information can be gained when the morphological changes are considered.

BHA. BHA at 2% in the diet induced carcinomas mainly in the prefundic region of the rat forestomach (12). In the present study, severe diffuse hyperplasia in this very region were found in all animals treated at this dose level. The midregion was generally less affected and the saccus caecus presented merely hyperkeratinization. Thus, the regional severity of the lesions clearly correlated with the localization of the tumors observed in the 2-year bioassay.

Takahashi and Hasegawa (27) described the sequential development of epithelial tumors in the forestomach of rodents, starting from
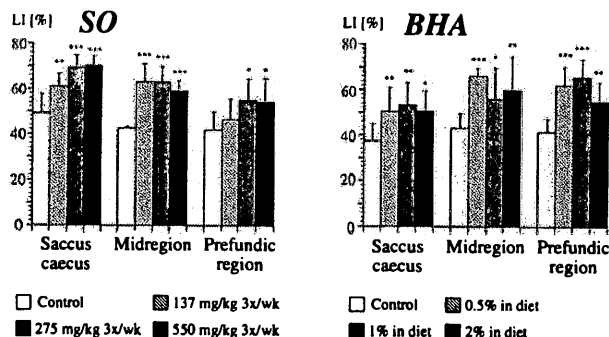

Fig. 2. Cell proliferation rates (LI) in three regions of the forestomach of male F344 rats following p.o. gavage of styrene oxide 3 times/week for 4 weeks (A) and BHA at 0–2% in the diet for 4 weeks (B). Mean values ± 1 SD. *, $P < 0.05$; **, $P < 0.025$; ***, $P < 0.0025$; 2-sided Fisher test.

epithelial damage to hyperplasia, acanthosis, hyperkeratosis and parakeratosis, followed by severe diffuse hyperplasia, dysplasia, papilloma and/or papillomatosis, and squamous cell carcinoma. The forestomach lesions observed in our BHA animals corresponded to those considered by Takahashi and Hasegawa to be early lesions in tumor development.

The hyperplastic lesions were quantitated in this study by the total number of cells along a defined section length. The results fully agreed with the histopathological findings: the more severe the lesions, the more cells were found. This finding is also in line with the concept of the "cells at risk" (3): the larger the number of division-competent cells, the higher the probability that one of them can take the next step towards malignant transformation.

SO versus BHA. When looking at carcinogenicity and mutagenicity data, there is a quite different activity profile of the two compounds. When given at the maximum tolerated dose for two years (SO, 550 mg/kg 3 times/week; BHA, 2% in the diet), SO induced squamous cell carcinomas in male rats with an incidence of 84% (11), whereas only 22% was reported for BHA (12). At one-half the maximum tolerated dose, SO induced forestomach carcinomas in as many as 67% of the rats, whereas 1% BHA in the diet solely gave rise to the formation of benign lesions, i.e., papillomas, in 20% of the animals, within 2 years. Comparable dosing regimens were used in this study. We therefore would have expected a generally stronger response of the SO-treated animals. With respect to hyperplasia, the opposite was true. Therefore, additional mechanisms of action must be taken into consideration to explain the high carcinoma incidence from SO.

SO was mutagenic in a number of short-term assays (13). It was shown to bind to DNA in vitro (28) although not detectably in vivo (14). The conclusion of the in vivo study was that a purely genotoxic mechanism of tumorigenesis was unlikely. This conclusion is supported by the findings of the present study: a clear increase is seen in the cell proliferation rate. For SO, therefore, both marginal genotoxicity by DNA binding and an enhanced rate cell proliferation play a role in the forestomach tumors induced in the bioassays.

A similar situation has been postulated by Cohen and Ellwein (29) for the bladder tumors induced by 2-acetylaminofluorene. While DNA-adduct levels in the bladder increased linearly with dose, an increase in the rate of cell division and the tumor incidence was seen only at higher dose levels. A multiplicative effect for the rate of cell division on the DNA adducts would nicely explain the strongly nonlinear dose-response for bladder tumors. A similar nonlinearity in the dose-response relationship could be expected for SO-induced forestomach carcinogenesis. The high-dose data cannot, therefore, be linearly extrapolated to low-dose levels despite the fact that SO has a genotoxic potential.

ACKNOWLEDGMENTS

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