Site-specific Cell Proliferation in Renal Tubular Cells by the Renal Tubular Carcinogen \textit{tris}(2,3-Dibromopropyl)phosphate

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Our laboratory has been examining the mechanisms whereby chemicals are mutagenic in short-term \textit{in-vitro} assays yet are not carcinogenic in 2-year rodent bioassays. Previous studies indicated that mutagenic carcinogens increased the amount of cell turnover in the target organ, but that mutagenic noncarcinogens failed to do so. The present study compares the incidence of cell proliferation in specific regions of the kidney, which is the site of carcinogenicity, with cell proliferation induced in a nontarget tissue, the liver, by the mutagenic renal tubular carcinogen \textit{tris}(2,3-dibromopropyl)phosphate (TRIS). Renal tubular adenocarcinoma induced by TRIS was the only tumor type identified in male F344 rats, and it was localized in the outer medulla. Male F344 rats were fed a diet containing 0, 50, or 100 ppm TRIS for 14 days. These doses were identical to the doses given in the National Toxicology Program cancer bioassay. Replicating cells were labeled with bromodeoxyuridine administered by an osmotic minipump and identified in tissue sections from liver and kidney using immunohistochemical techniques. Examination of liver sections showed no chemically related increases in cell proliferation above control for either dose group. However, in the kidney, TRIS induced significant cell proliferation that was localized in the renal outer medulla region, the target area for carcinogenesis. The labeling index (number of labeled cells/total number of cells counted) in the kidneys of TRIS-exposed rats was increased approximately 4-fold in the outer medulla and was not increased in the cortex or inner medulla. The results of this study suggest an association between the chemically-induced renal cell proliferation and the renal carcinogenicity of TRIS.

Introduction

Recent reports\textsuperscript{(1,2)} have demonstrated that although there is a high degree of association between mutagenesis in Salmonella and carcinogenicity in rodents, there are also a large number of carcinogens whose actions are not explained by mutagenicity and structural alert data. Our studies center on mutagenic noncarcinogens and describe comparisons with mutagenic carcinogens in an effort to discover the mechanism(s) whereby the carcinogens act to produce tumors that are missing in the effect of the noncarcinogens. The mutagenic noncarcinogens are the class of chemicals referred to as "false-positives," chemicals that produce mutagenicity in short-term assays and possess structural alerts\textsuperscript{(3)}, which would, therefore, be predicted to be carcinogenic, yet are noncarcinogenic in rodents. Results presented here represent a continued effort to determine the sources of these apparently discordant results and are of value in understanding the differences between \textit{in-vitro} and \textit{in-vivo} systems for the evaluation of chemical carcinogenicity or lack thereof.

Our previous results indicate that the carcinogenicity of a chemical may be related not only to its DNA-damaging capability as measured by its activity in mutagenicity tests, but also to its ability to cause an increase in cell division in animals\textsuperscript{(4,5)}. The flame retardant \textit{tris}(2,3-dibromopropyl)phosphate (TRIS; Fig. 1) was studied to examine the cell proliferative effects of a mutagenic renal carcinogen. TRIS is mutagenic in the Ames/Salmonella assay, in the presence of

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metabolic activation (6). TRIS was demonstrated to be a potent renal carcinogen in male and female F344 rats and male B6C3F1 mice (7). A closer examination of the lesions indicated that TRIS caused renal tubular adenoma and adenocarcinoma primarily in the outer medulla. Results of the present study provide further evidence supporting the role of cell proliferation in carcinogenesis.

Materials and Methods

Animals

Male Fischer F344 rats (Charles River Breeding Laboratories, Raleigh, NC) weighing 150 g were maintained on a standard NIH 31 diet ad libitum and a daily cycle of alternating 12-hr periods of light and darkness. The animals were acclimated to this environment for 2 weeks before the experiment began and were maintained at 21-23°C and at 40-60% relative humidity. Rats were randomly assigned to treatment groups of 5 animals per group and allowed 24 hr to adapt to a new cage environment and the powdered chow mixture (without chemical), described below. Experiments were performed according to the guidelines established in the NIH Guide for the Care and Use of Laboratory Animals.

Chemical Treatment

Wy-14,643, a potent hepatocarcinogen, was used in this study as a positive control for increased hepatic cell proliferation. Wy-14,643 was purchased from Chemsyn Science Laboratories (Lenexa, KS). TRIS was obtained from the National Toxicology Program NTP repository. Chemicals were dissolved in acetone (100 μL) and added to a mixture of powdered NIH 31 chow (20 g/rat) and agar (2 g/rat), followed by addition of warm water (60 mL/rat). The final concentration of Wy-14,643 was 100 ppm and TRIS was 50 or 100 ppm. This mixture was a moist solid and was very palatable to the rats. It was prepared daily for each animal and was their sole source of food during the experiment. Control animals received this powdered chow mixture with 100 μL acetone. Animals were killed 1 day after the final dose.

Cell Proliferation Measurements

The animals were allowed to adjust to chemical treatment for 1 day. Osmotic minipumps (Alza Corporation, Palo Alto, CA, model 2002) were then implanted subcutaneously into the backs of the rats. These minipumps delivered bromodeoxyuridine (BrdU) (Sigma Chemical Co., St. Louis, MO) at 30 μg/hr for 2 weeks. BrdU is incorporated into the DNA of replicating cells. Fourteen days later, the animals were euthanized by CO2 inhalation, livers were weighed, and mid-lobe radial sections of the right anterior lobe were fixed in 70% cold ethanol for 24 hr. The mid-lobe radial section was chosen to avoid possible interlobe variation in BrdU incorporation. A cross-section of small intestine was also fixed as a positive control for the proper operation of the minipump and the staining technique. Tissues were embedded in paraffin, stained with hematoxylin and eosin for histological analysis, and stained immunohistochemically for BrdU incorporation as described previously (4,5). Random areas of the slides were chosen for counting stained and unstained hepatocyte nuclei (>1000 hepatocytes/rat). Because Wy-14,643 produced an uneven distribution of labeling across the lobule, the labeling index included labeled and unlabeled hepatocytes in periportal, midzonal, and centrilobular areas (approximately 350 hepatocytes per area). Three counts from each area were made from each animal. Statistics were performed using the Student’s t-test and summed for comparison to the vehicle-treated control. Kidneys in some experiments were perfused briefly with sodium phosphate buffer (pH 7.4) followed by 2% paraformaldehyde-1% glutaraldehyde (8). This allowed for analysis of cell proliferation in specific segments. Our preliminary results indicated that there was no difference in labeling between P1, P2, or P3 segments. Therefore, kidneys were transected into 2-mm sections (median transverse) and drop fixed in 10% neutral buffered formalin for 24 hr, followed by paraffin embedding and sectioning at 2 μm, followed by immunohistochemical staining as described. Quantitation of cell proliferation was performed as described by Short et al. (8), except that quantitation of labeling in specific segments was not necessary.

Results

Rats exposed to TRIS showed no changes in liver/body weight ratios compared to vehicle-treated animals. The positive control, Wy-14,643 showed a statistically significant increase in this ratio (Table 1). Hepatocyte proliferation, as measured by BrdU incorporation in livers of rats treated with TRIS, was not statistically different from vehicle-treated animals (Table 2). Cell proliferation in the livers of animals exposed to dietary Wy-14,643 showed an approximately 13-fold increase in cell proliferation during the course of the 2-week exposure period (Table 2).
Table 1. Weight gain and liver/body weight ratios for male F344 rats after dietary exposure to tris(2,3-dibromopropyl)phosphate (TRIS) or Wy-14,643 for 14 days.

| Treatment          | Body weight gain, g | Liver weight/body weight |
|--------------------|---------------------|--------------------------|
| Vehicle (acetone)  | 9.0 ± 7             | 0.044 ± 0.004            |
| Wy-14,643, 100 ppm | 0.6 ± 9             | 0.076 ± 0.004*           |
| TRIS               |                     |                          |
| 50 ppm             | 12.4 ± 1            | 0.049 ± 0.001            |
| 100 ppm            | 11.7 ± 1            | 0.046 ± 0.001            |

*p < 0.01 compared to vehicle control; n = 5.

Table 2. Bromodeoxyuridine labeling indexes of hepatocytes in male F344 rats after dietary exposure to tris(2,3-dibromopropyl)phosphate (TRIS) or Wy-14,643 for 14 days.

| Treatment          | Labeling index, %a | Fold increase over control |
|--------------------|--------------------|----------------------------|
| Vehicle (acetone)  | 3.4 ± 1            |                            |
| Wy-14,643, 100 ppm | 65 ± 12*           | 19*                        |
| TRIS               |                    |                            |
| 50 ppm             | 3.0 ± 1.0          | 0.9                        |
| 100 ppm            | 2.8 ± 0.3          | 0.8                        |

*aNumber of hepatocytes staining positive for bromodeoxyuridine/1000 hepatocytes × 100.
*bAverage of counts from periportal, midzonal, and centrilobular areas.
*p < 0.001 compared to vehicle control; n = 5.

Because Wy-14,643 induces primarily a periportal pattern of cell proliferation by this exposure regimen, measurements from both periportal and centrilobular areas were averaged. The only group demonstrating microscopic changes was the Wy-14,643 group. Livers of rats in this treatment group exhibited slightly enlarged hepatocytes compared to controls, and the cytoplasm was more eosinophilic, with a fine granular appearance.

Only kidneys from animals treated with TRIS showed increased cell proliferation (Table 3; Figure 2). The 14-day exposure to 50 ppm TRIS increased the labeling index in the outer medulla 4.0-fold above the control value. Labeling indexes in the cortex of inner medulla were not significantly affected, nor were there increases in the labeling indexes of rats treated with Wy-14,643. Increased cell proliferation in the outer medulla was also observed in the high-dose group (100 ppm), but it did not appear to be dose related (Table 3). No other microscopic changes were observed in the kidneys of either the Wy-14,643 or the TRIS-treated animals.

Discussion

Cell proliferation is known to be an important component in the induction of chemical carcinogenesis in multistage rodent carcinogenesis models (9). Recent studies have demonstrated a strong correlation between chemically induced cell proliferation and carcinogenesis by a variety of genotoxic (4,5) and non-genotoxic compounds (10). Although the exact role of induced cell proliferation in carcinogenesis is not certain (11), increasing the rates of cell division, either by mitogenesis or compensatory hyperplasia secondary to cell killing, may prevent the repair of spontaneously occurring mutations (12) or activate protooncogenes (13). Data in this report support our earlier speculation that chronic, chemically induced toxicity and resulting cell proliferation may play a role in carcinogenesis induced by mutagenic chemicals.

The observation that TRIS induced cell proliferation only in the outer medulla of the kidney prompted a further review of the slides of the TRIS bioassay. Results of this evaluation indicated that renal adenomas and adenocarcinomas were found almost exclusively in the outer medulla after 2-year feeding studies and provide strong evidence for the role of cell proliferation in the renal carcinogenesis induced by TRIS. The observation that cell proliferation may be tissue or cell-type specific may, therefore, be of predictive value. Information on the mutagenicity of a chemical, in addition to data on the site of induction of cell proliferation, may prove useful for the prediction of carcinogenicity and its localization within a tissue or organ. The observation that the dose-response relationship for cell proliferation in this study and the incidence of renal tubular carcinogenesis were both flat is very interesting. This suggests that at 50 ppm, a maximal effect occurred in both studies. This may have resulted from saturated absorption or metabolism of TRIS, and preliminary (unpublished) data indicate that it is a gender-related phenomenon. Further studies are being conducted on the toxicokinetics of TRIS to more fully explain this observation.

The experimental design in this study was intended to examine the early effects of chemical exposure on
cell replication. It was not intended to answer the question of the sustainability of the induced cell replication. Transient bursts of cell proliferation, up to several weeks, may be sufficient to produce the conditions favorable to but may not be sufficient for induction of carcinogenesis (14). This may be especially true for mutagenic chemicals, and the combination of mutagenicity and at least a transient round of cell replication may enhance the induction of carcinogenesis (4).

In support of this concept, it was observed that the first identifiable step in diethylnitrosamine hepatocarcinogenesis is the appearance of altered foci containing glutathione transferase-placental form (GST-P*), which was demonstrated to occur in the same hepatocytes that stain positive for BrdU (15). These data suggest that induced cell proliferation may act in the initiation stage of chemical carcinogenesis, at least for mutagenic chemicals, as has been observed earlier (9). Nonmutagenic carcinogens, such as Wy-14,643, may require more sustained levels of cell proliferation to induce carcinogenesis than are required for mutagenic carcinogens (14). Clearly, more research is needed to evaluate the relevance of transient versus sustained cell proliferation in the carcinogenicity of mutagenic and nonmutagenic chemicals.

Cell proliferation data may become routine in the evaluation of chemical toxicity, and we are incorporating the evaluation of cell proliferation into many of the NTP toxicity studies. As the relationship of cell proliferation and carcinogenesis appears to be tissue, cell type, and possibly species and gender specific, extrapolation of these effects will require additional research.

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