Carcinogenicity in Rats of the Nitrosated Bile Acid Conjugates
N-Nitrosoglycocholic Acid and N-Nitrosotaurocholic Acid

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ABSTRACT

Two nitrosated bile acid conjugates, N-nitrosoglycocholic acid and N-nitrosotaurocholic acid, were examined for carcinogenicity in a 2-year study with male Fischer rats using a 6-week p.o. dosing protocol with a total of 300 mg compound/rat. Both compounds were approximately equally carcinogenic and induced significant levels of hepatocellular carcinoma in 54 to 70% of the animals at risk. Gastric tumors of the glandular and antral stomach were observed in 12 to 13% of the treated rats. Although the incidence was not significant, these levels were much higher than those in historic controls. Malignant liver and gastric tumors were not detected in vehicle control rats. Alkaline phosphatase-positive foci, putative early mucosal alterations which may precede neoplasia, were found in approximately 35% of the glandular stomachs of compound-treated rats but not in those of control rats.

INTRODUCTION

The existence of N-nitroso compounds in the environment and their implications for human cancer risk have been well documented (9, 10, 18, 19). Although much of the research in this area has been directed towards formation and biological activity of the N-nitrosamines, less effort has focused upon the N-nitrosamides which are known to be potent direct-acting mutagens and carcinogens. Many N-nitrosamides including methyl-

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4 The abbreviations used are: i.g., intragastric; DMSO, dimethyl sulfoxide; NOGC, N-nitrosoglycocholic acid; NOTC, N-nitrosotaurocholic acid; PSA, Pollock or Mantel-Cox analysis of data, respectively.

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CARCINOGENICITY OF NITROSATED BILE ACID CONJUGATES

Chart 1. Structures of NOGC (I) and NOTC (II).

(total dose, 300 mg in 1.5 ml DMSO). The control group received DMSO only.

At the end of the dosing period, 4 animals were sacrificed from the control and each treatment group for gross and histopathological evaluation. The remaining rats were housed without further treatment for up to 112 weeks of age when significant morbidity among the treated animals necessitated termination of the experiment.

Rats were killed by CO₂ asphyxiation and subjected to complete necropsy. The stomachs were opened along the greater curvature and pinned flat on cardboard prior to fixation, along with other tissues, in buffered formalin.

Histopathology. The formalin-fixed stomachs were examined for the presence of alkaline phosphatase-positive foci, considered to be an indicator of the later development of intestinal metaplasia, according to the procedure of Morgan and associates (14).

The liver, stomach, tissue masses, and other grossly abnormal organs were trimmed, sectioned, stained with hematoxylin-eosin, and subjected to histopathological examination. Special care was taken to include any alkaline phosphatase-positive foci in the preparation of the stomach sections.

Rats with liver lesions were tabulated only once according to the most severe lesion in the following order: hepatocellular carcinoma > adenoma > hyperplastic nodule.

RESULTS

General Observations. No significant differences were noted in animal weight gains during the 6-week dosing period between control and NOGC- or NOTC-treated rats. The average weight of the rats was approximately 60 g at the start of dosing and 205 g at the end resulting in a daily dose varying from 167 to 49 mg nitrosated bile acid/kg body weight during the course of treatment. The average total dose for each compound was approximately 2150 mg/kg body weight. Gross and histopathological examination of tissues from rats sacrificed at the end of the dosing period did not reveal any abnormalities.

Sixty-five % of the DMSO controls remaining after treatment and the interim sacrifice survived to the terminal sacrifice at 112 weeks. No hepatocellular carcinomas were observed in these animals, but 2 instances of lymphoma and one of lung adenocarcinoma were diagnosed during the 47- to 112-week period. The comparable survival rate for NOGC- and NOTC-treated rats was 48 and 56%, respectively. Most of the premature deaths in these treatment groups occurred in animals with hepatocellular carcinoma. One leiomyosarcoma of the stomach (80 weeks) and an ear duct squamous cell carcinoma (97 weeks) were also recorded in NOGC-treated rats, and a lymphoma (99 weeks) and a squamous cell carcinoma of the stomach (107 weeks) were observed in NOTC-treated animals.

Liver Tumors. The cumulative probability of death from hepatocellular carcinoma over the course of the experiment was calculated by the product-limit (Kaplan-Meier) estimate (3), and the data are presented in Chart 2. The first hepatocellular carcinoma was observed at 47 weeks of age in the NOGC group and at 80 weeks in the NOTC group. The plot for NOGC was significantly different from that of the DMSO control (pₐ = 0.10; pₑₐₐₙ = <0.01), whereas the plot for NOTC was only marginally different (pₑ = 0.054; pₑₐₐₙ = 0.053). There was no difference between the NOTC and NOGC plot (pₑ = 0.250; pₑₐₐₙ = 0.271).

The incidence of malignant and benign tumors from this study is shown in Table 1. The major finding is the significant elevation of hepatocellular carcinomas in the NOTC (54%) and NOGC (70%) treatment groups as compared to the absence of these tumors in DMSO controls. There was no significant difference in hepatocellular carcinoma incidence between the NOGC and NOTC groups; the 2 compounds therefore appear to have approximately the same potency as carcinogens. The gross and microscopic appearance of typical liver tumors are shown in Figs. 1 and 2.

Hyperplastic nodules of the liver were detected at essentially the same incidence in all rats, although a marked increase in size and number of these lesions was readily apparent only in animals receiving NOGC and NOTC. A low incidence of hepatocellular adenomas (approximately 10%) was found only in the nitrosated bile acid treatment groups, and benign tumors of the bile duct (cholangiomas) occurred only in NOGC-treated rats.
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Stomach Tumors. Although benign and malignant stomach tumors were not observed in control animals, they were recorded in animals treated with NOTC or NOGC at a statistically insignificant incidence of 12 and 13%, respectively (Table 1). There was no apparent relationship between treatment and site of the tumors since there were examples from both the glandular (adenocarcinoma) and aglandular (squamous cell carcinoma) portions of the stomach. With the single exception of one gastric adenocarcinoma in an NOTC-treated rat, the other 4 malignant stomach tumors were detected only upon microscopic examination.

Glandular hyperplasia of the stomach mucosa was diagnosed in 35 to 45% of the rats in each of the control and treatment groups. There was a lesser incidence (15 to 20%) of gastritis and ulcercation, which occurred in the aglandular portion of the stomach. These lesions therefore were seemingly not associated with exposure to NOGC or NOTC.

Micrographs of stomach sections illustrating features of squamous cell carcinoma, mucosal hyperplasia, and adenocarcinoma are shown in Figs. 3 to 6.

A variety of tumors at sites other than the liver and stomach were also present in the control and treated rat groups and are listed in Table 1. The majority of these tumors were lymphomas and occurred in all animal groups. There was no indication of trends or statistical significance with regard to these miscellaneous tumors. It is noteworthy that tumors were not found in the duodenum or any other portion of the gastrointestinal tract.

Intestinal Metaplasia. The presence of alkaline phosphatase-positive foci as a possible histochemical marker of early intestinal metaplasia in stomachs clearly showed a correlation with nitrosated bile acid treatment. Approximately 35% (NOTC, 35%; NOGC, 36%) of the stomachs from the treated rats exhibited phosphatase activity, whereas it was not detected in control rats. The majority of the positive foci were located in the antral mucosa. There did not, however, appear to be any connection between these foci and the presence of glandular hyperplasia or gastritis.

Discussion

The results of this study clearly indicate that NOGC and NOTC are hepatocarcinogens in the rat following a 6-week p.o. dosing regimen. To our knowledge, this is the first demonstration that nitrosated derivatives of naturally occurring amides are carcinogenic.

No significant differences in hepatocarcinogenicity were noted between essentially equivalent doses of NOGC and NOTC, although a higher incidence of hepatocellular carcinoma was evident in the NOGC treatment group (70 versus 54%). Animals treated with NOGC also appeared to develop hepatocellular carcinoma more rapidly with a higher probability of death. No

Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>No. of rats</th>
<th>Tumors</th>
<th>Stomach tumors</th>
<th>Other tumors</th>
<th>Total tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Malignant (hepatocellular carcinoma)</td>
<td>Benign</td>
<td>Hyperplastic nodules</td>
<td>Malignant</td>
</tr>
<tr>
<td>DMSO</td>
<td>18</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>5 (28)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>NOTC</td>
<td>24</td>
<td>13 (54)</td>
<td>3 (12)</td>
<td>5 (21)</td>
<td>2 (8)</td>
</tr>
<tr>
<td>NOGC</td>
<td>23</td>
<td>16 (70)</td>
<td>2 (9)</td>
<td>4 (17)</td>
<td>3 (13)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage.
* Two lymphomas; 1 mesenteric lymphosarcoma; 1 lung adenocarcinoma plus a squamous cell carcinoma of the skin.
* One mammary gland adenoma.
* Significantly different from DMSO controls, at p < 0.01.
* One squamous cell carcinoma; 1 adenocarcinoma.
* One papilloma.
* Three lymphomas.
* One mammary gland adenoma; 1 keratoacanthoma.
* Significantly different from DMSO controls, at p < 0.03.
* Two cholangiomas.
* One squamous cell carcinoma; 1 adenocarcinoma; 1 leiomyosarcoma (aglandular stomach).
* One lymphoma; 1 ear duct squamous cell carcinoma; 1 s.c. leiomyosarcoma.
* Significantly different from DMSO controls, at p < 0.002.

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difference was apparent with respect to benign liver adenomas and hyperplastic nodules in the NOGC and NOTC treatment groups.

Both nitrosated bile acid conjugates were bacterial mutagens, being about equally potent in forward mutation and reversion assays in Salmonella typhimurium (22). NOGC was, however, a potent mutagen in a diploid human lymphoblast assay, being approximately 9000 times more potent than NOTC, although cytotoxicity of these 2 compounds was comparable. Thus, this differential response in the human cell mutation assay was not apparent in the rat carcinogenicity bioassay.

The major rationale for testing the nitrosated bile acid conjugates for carcinogenicity was to determine whether the stomach (particularly the glandular portion) was a target organ. It was, therefore, of interest that the incidence of the observed stomach tumors was not statistically significant. However, tumors from both the glandular and aglandular stomach were observed only in the NOTC and NOGC treatment groups at incidences (12 and 13%) many times higher than those spontaneously observed in the male Fischer rat. The incidence of stomach tumors in over 8000 historical control Fischer rats of both sexes in the National Cancer Institute/National Toxicology Program 2-year carcinogenesis bioassays was sufficiently low (<2%) to preclude listing in the summary by Haseman (7). Goodman et al. (6) reported malignant gastric tumors in only 4 of 1794 (0.2%) and 3 of 1754 (0.2%) control male and female Fischer rats, respectively. Three of these tumors (43%) were squamous cell carcinomas originating in the glandular stomach, 2 (29%) were glandular stomach tumors (adenocarcinoma and basal cell carcinoma), and the remaining 2 tumors (29%) were sarcomas of unstated origin. Spontaneous gastric tumors were also extremely rare (1 tumor in 1464 animals) in a separate compilation of other life span studies with control Fischer rats (20).

The fact that mucosal hyperplasia of the glandular stomach and gastritis and ulceration of the aglandular stomach were observed in a substantial number of control and treated rats may indicate that some degree of mechanical trauma was sustained during dosing. This trauma may have predisposed the animals to the induction of stomach tumors following concurrent exposure to the direct-acting nitrosamines, NOTC and NOGC. This is supported to some degree by other observations of a comparatively low incidence of ulceration (0.8%) and squamous cell hyperplasia (1.5%) in stomachs of aging control male Fischer rats (6). On the other hand, there was no evidence of gross or histopathological damage to the stomach in rats sacrificed immediately following the dosing period. Further studies in which rats would be dosed with compound administered in the drinking water would be necessary to eliminate the possibility that trauma associated with dosing by stomach tube effected tumor formation.

The presence of alkaline phosphatase-positive foci in the antral mucosa of the stomach as an indicator of intestinal metaplasia was clearly associated with treatment with NOGC and NOTC. The lack of correlation with glandular hyperplasia or gastritis indicated that functional changes in alkaline phosphatase activity occurred before histological changes were detectable. Although it has not been established that either alkaline phosphatase activity or intestinal metaplasia is indicative of the preneoplastic state, epidemiological and experimental studies have indicated that gastric adenocarcinoma and intestinal metaplasia are developmentally related (2).

ACKNOWLEDGMENTS

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REFERENCES

Fig. 1. Gross appearance of a multicentric hepatocellular carcinoma typical of those observed in the NOTC- and NOGC-treated rats. All lobes were involved with nodules of tumors varying from small foci to confluent nodules 2 to 3 cm in diameter.

Fig. 2. Trabecular carcinoma typical of the liver tumors encountered in this study. H & E, × 250.

Fig. 3. Invasive squamous cell carcinoma of the forestomach observed in a NOGC-treated rat. A segment of the squamous epithelium can be observed at the bottom. H & E, × 100.

Fig. 4. Glandular hyperplasia of gastric mucosa observed in NOTC- and NOGC-treated rats. These focal areas, which appeared to have no zonal preference, probably preceded neoplastic changes. H & E, × 250.

Fig. 5. Low-power view of an adenocarcinoma of the glandular stomach from a NOTC-treated rat. There is invasion of tissue in the stalk and at the base of the polypoid tumor. H & E, × 25.

Fig. 6. High-power view of the gastric adenocarcinoma. Note irregular glandular structures filled with mucous secretion, nuclear pleomorphism, reactive cell infiltrate in the stroma, and frequent mitotic figures. H & E, × 400.
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