A Comparative Immunohistochemical Study of Spontaneous and Chemically Induced Pheochromocytomas in B6C3F1 Mice

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Abstract

Spontaneously occurring and chemically induced pheochromocytomas are rare in mice. That the mouse pheochromocytoma is a more appropriate animal model than that of the rat for study of human medullary adrenal tumors has been suggested. The expression of phenylethanolamine-N-methyltransferase (PNMT), the enzyme responsible for production of epinephrine from norepinephrine, is common to both mouse and human pheochromocytomas. This investigation assessed the expression of the immunohistochemical markers PNMT, tyrosine hydroxylase (TH), and chromogranin A (CGA) in spontaneously occurring and chemically induced pheochromocytomas in the B6C3F1 mouse. Spontaneous tumors were derived from control animals from 10 different studies and the pheochromocytomas from treated groups from 4 different studies. All tumors were positive for maximal TH expression. A highly significant difference in PNMT expression ($p < 0.01$) occurred between spontaneously occurring pheochromocytomas classified as benign or "malignant" by the criteria of toxicologic pathology. Chemically induced tumors showed intermediate PNMT staining. A marked reduction in CGA expression occurred in pheochromocytomas induced by technical grade pentachlorophenol, compared to the other three chemicals and the spontaneously occurring tumors. These findings suggest that immunohistochemistry is a reliable tool in investigating the functional capabilities of pheochromocytomas in mice. PNMT expression is a tightly regulated component of the chromaffin cell phenotype and appears to be readily lost in mouse pheochromocytomas, particularly those with aggressive characteristics.

Key Words: Pheochromocytoma; spontaneous; chemically induced; immunohistochemistry; B6C3F1 mouse.

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Introduction

Spontaneous and chemically induced pheochromocytomas in mice occur rarely with reported lifetime frequencies of ≤ 3% in most control group studies [7,11,17–19]. They appear in several different strains of mice including B6D2F1, B6C3F1, and C57BL6 [17]. That these neoplasms have morphological features similar to those from humans has been reported [4] and suggests their use as an appropriate model for human adrenal medullary tumors [12,17]. As in human pheochromocytomas, spontaneously occurring pheochromocytomas in mice stain variably positively for phenylethanolamine-N-methyltransferase (PNMT), the enzyme that mediates the production of epinephrine from norepinephrine [17].

There are several reported differences between the more extensively studied rat pheochromocytomas and those that arise in the mouse. First, both spontaneously occurring and xenobiotic-induced pheochromocytomas occur more commonly in rats [6] than mice. The highest incidence of pheochromocytomas in mice is less than one-tenth of that occurring in many strains of rats [17,19]. In addition, expression of immunoreactive PNMT is an uncommon finding in pheochromocytomas of rats [17,20,21] suggesting that functional differences may exist between these neoplasms and those found in mice and in humans. In contrast to rats, few chemicals have been reported to cause mouse adrenal medullary tumors. A recently published compendium of chemical carcinogens listed by target organs noted eight chemicals that caused adrenal tumors in mice [5]. These included carbon tetrachloride; furan; 4,4'-methylenedianiline-2 hydrochloride; pentachloroanisole; 2,3,4,5,6-pentachlorophenol (Dowicide EC-7); 2,3,4,5,6-pentachlorophenol, technical grade; *p*-rosaniline HCl; and 1,1,2-trichloroethane. The incidence of spontaneous pheochromocytomas is higher in male rats than in females; however, the incidence is similar between male and female mice [7]. Pheochromocytomas are usually unilateral in mice, but very often bilateral in the rat [11]. The National Toxicology Program (NTP) has published technical reports of 514 compounds with 7 associated with pheochromocytomas in mice and 13 in rats (http://ntp-server.niehs.nih.gov/).

Immunohistochemical procedures have been used to correlate functional and morphologic features in spontaneously occurring pheochromocytomas in mice [15]. These tumors were positive for tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis, and variably positive for both PNMT and chromogranin A (CGA), a granule matrix constituent reflecting the number of secretory granules that a chromaffin cell contains [17,20]. Immunohistochemical studies have not been reported in chemically induced pheochromocytomas.

Over the past few decades, the NTP has conducted seven chronic bioassays in mice that have led to chemically induced pheochromocytomas. Four of these studies were chosen for immunohistochemical evaluation to determine if there were functional differences between these tumors.

Materials and Methods

Histological Examination

The NTP conducts 2-yr carcinogenesis studies generally in the B6C3F1 mouse and in the F344 rat. These studies are conducted at various contract laboratories throughout the United States and usually include 50 animals/dose/sex/species. Following complete gross evaluation at necropsy and histological assessment of routine organs and every lesion, pathology tables are generated to summarize neoplastic and nonneoplastic diagnoses.

For the current study, 14 pheochromocytomas from control male and female and 27 pheochromocytomas from treated male B6C3F1 mice were retrieved from 2-yr NTP carcinogenicity studies from the last 20 yr (Tables 1 and 2, respectively). All mice were between 109 and 117 wk of age at the time of sacrifice, and they had no concurrent endocrine neoplasms. The spontaneous tumors were detected in control animals from 10 different studies, and the pheochromocytomas in treated groups were derived from 4 different studies. These four chemicals (Table 3) were furan, 4,4'-methylenedianiline, Dowicide pentachlorophenol, and technical grade pentachlorophenol (TGP). Tumors were classified

Table 1. Pheochromocytomas in Control Mice from NTP 2-Yr Carcinogenicity Studies

Compound/TR#	No. of mice with tumors	Route of exposure	No. of "malig" /benign	Sex
Acetonitrile/447	2	Inhalation	1 "malig"	1F
			1 benign	1F
Barium chloride dihydrate/432	1	Water	"malig"	F
1-Chloro-2-propanol/477	1	Water	"malig"	F
Citral/505	1	Feed	"malig"	F
1,2-Dihydro-2,2,4-trimethylquinoline/456	3	Dermal	1 "malig"	1F
			2 benign	1F, 1M
Emodin/493	1	Feed	"malig"	F
Nickel oxide/451	1	Inhalation	benign	F
Riddelliine/508	1	Gavage	"malig"	M
Scopolamine hydrobromide/445	1	Gavage	"malig"	F
Tricresyl phosphate/433	2	Feed	2 benign	1F, 1M

Data from the NTP Technical Reports (TR) are available from the NTP, NIEHS and at http://ntp-server.niehs.nih.gov/.

Table 2. Pheochromocytomas in Treated Male Mice from NTP 2-Yr Carcinogenicity Studies

Compound/TR#	No. of mice with tumors	Route of exposure
Furan/402	9	Gavage
4,4'-Methylenedianiline/248	5	Water
Dowicide pentachlorophenol/349	5	Feed
Technical grade pentachlorophenol/349	8	Feed

Data from the NTP Technical Reports (TR) are available from the NTP, NIEHS and at http://ntp-server.niehs.nih.gov/.

Table 3. Use, Genetic Toxicity, and Carcinogenicity of Chemicals Associated with Pheochromocytomas in Male Mice from NTP 2-Yr Studies

Compound/TR#/Use/Genetic Toxicity (GT)/Carcinogenicity
Furan/402
Use: intermediate for polymer synthesis
GT: induce gene mutations, sister chromatid exchanges, and chromosomal aberrations
Carcinogenicity: treatment-related neoplasms in liver and adrenal gland
4,4'-Methylenedianiline/248
Use: polyurethane intermediate
GT: induce gene mutations and sister chromatid exchanges [2,14]
Carcinogenicity: treatment-related neoplasms in thyroid, liver, and adrenal gland
Dowicide pentachlorophenol/349
Use: wide-spectrum biocide
GT: induce sister chromatid exchanges and chromosomal aberrations
Carcinogenicity: treatment-related neoplasms in liver and adrenal gland
Technical grade pentachlorophenol/349
Use: wide-spectrum biocide
GT: induce sister chromatid exchanges and chromosomal aberrations
Carcinogenicity: treatment-related neoplasms in liver and adrenal gland

Data from the NTP Technical Reports (TR) are available from the NTP, NIEHS at http://ntp-server.niehs.nih.gov/.

as benign or malignant according to the criteria of toxicologic pathology [11,12], in which tumors with aggressive characteristics (i.e., obliteration of the cortex, capsular penetration, or blood vessel invasion) are considered "malignant" with or without documented metastases. "Malignant" pheochromocytomas were found exclusively in the control animals. A gender comparison was not evaluated in this study; however, spontaneous pheochromocytomas appeared to occur more frequently in female mice, while the chemically induced tumors were more prevalent in male mice. These mice were not evaluated for hypertension or serum catecholamine levels.

Adrenal glands were fixed by immersion in 10% buffered formalin, dehydrated in a graded ethanol series, and embedded in paraffin wax. Serial sections were cut 4–5 μm thick and either stained with hematoxylin and eosin (H&E) or used for immunohistochemistry.

Immunohistochemical Staining

Immunohistochemical reactions (Table 4) were performed using the biotin-avidin/peroxidase detection method [8] with the following mouse monoclonal (mAb) and rabbit polyclonal (pAb) antibodies: (1) anti-TH mAb, (2) anti-CGA pAb, and (3) anti-PNMT pAb.

All sections were deparaffinized in xylene, rehydrated through graded alcohol, placed in a 0.02 *M* boric acid solution (pH 7.0) and heated 2 min in a pressure cooker followed by a 20-min cooling period under pressure. All slides were incubated in 10% normal goat serum for 10 min. The immunohistochemical reactions for CGA and PNMT were carried out in the Ventana NexES immunostainer by using the biotinylated Ig/avidin-HRPO method (Ventana Strassbourg, France). For TH immunostaining, the Vector Mouse on Mouse kit was used (Vector Laboratories, Inc., Burlingame, CA). Endogenous peroxidase activity was blocked using an endogenous biotin blocking kit. Sections of normal mouse adrenal gland were used as positive controls for each of the antibodies used. Normal mouse serum substituted for anti-TH, and normal rabbit serum substituted for anti-CGA and anti-PNMT served as negative controls.

All pheochromocytomas were assessed for PNMT, TH, and CGA expression by comparing the staining of spontaneously occurring benign with spontaneously occurring "malignant" and chemically induced tumors. The extent of immunoreactivity for PNMT, TH, and CGA was scored by subjective assessment of the neoplasms using a five-point grading scale determined by using the percentage of tumor cells that stained for the particular antibody: 0–negative (0% staining of tumor cells), 1–minimal (up to 10% of tumor cells staining), 2–mild (up to 25% of tumor cells staining), 3–moderate (up to 60% of tumor cells staining), and 4–marked (>60% of tumor cells staining).

The Mann–Whitney *U* test was used to compare the immunohistochemical grading for PNMT, TH, and CGA. These comparisons were made among spontaneous and chemically induced benign and "malignant" pheochromocytomas. Data from 3 male and 11 female spontaneous pheochromocytomas were compared with those from the 27 chemically induced

Table 4. Antibody (Ab), Dilution, and Supplier

Primary Ab	Dilution	Supplier
Chromogranin A (CGA)	1:300	Dako Diagnostic, Zug, Switzerland
Tyrosine hydroxylase (TH)	1:1000	Zymed Laboratories, Inc., San Francisco, CA, USA
Phenylethanolamine-N-methyltransferase (PNMT)	1:1200	DiaSorin, Stillwater, MN, USA

pheochromocytomas in male mice. Comparisons were based upon the assumption that there was no sex difference in response.

Results

Histological Examination

Benign pheochromocytomas were primarily characterized by expansile cords or clusters of neoplastic cells often arranged in variably sized nodules that compressed the adjacent parenchyma. These tumor cells had a granular, basophilic cytoplasm. Cellular pleomorphism was prominent and mitotic figures were occasionally present (Figs. 1A, 3A, 4A). Pheochromocytomas were designated "malignant" if neoplastic cells obliterated the cortex, penetrated the capsule, invaded blood vessels, or metastasized (Fig. 2A). In this retrospective study, eight "malignant" pheochromocytomas were diagnosed (ranging in size from 0.7 to 14 mm) with two metastasizing to the lung.

Immunohistochemical Evaluation

All of the pheochromocytomas were given a grade 4 for TH immunoreactivity and were not evaluated statistically (Figs. 1D, 2D, 3D, 4D). This finding indicated the ability of the tumors to synthesize catecholamines. In pheochromocytomas that had multiple nodules, the staining characteristic for each nodule was taken into consideration when grading (Figs. 3B, 3C, 4B, 4C). If uniform staining occurred among nodules, one grade was given. If there were variations in staining among nodules, each nodule was assigned a grade, and the median grade was used in the statistical evaluation. In addition, the intensity of the immunohistochemical stain was not used when evaluating the pheochromocytomas owing to the variability between stains.

A highly significant ($p < 0.01$) difference in the grading score for PNMT existed between spontaneously occurring benign and "malignant" tumors (Table 5). There was no PNMT expression (grade 0) by the majority of the spontaneous "malignant" pheochromocytomas (Fig. 2B), but variable PNMT expression (grade 2.5) by spontaneous benign tumor cells (Fig 1B). Because there were no significant differences in the grading among the four chemical groups, their scores were pooled and an intermediate staining response (grade 1) was observed. These results confirm the presence of epinephrine-producing neoplastic cells in the spontaneous benign and chemically induced benign pheochromocytomas.

Variability in CGA staining was evident in the four groups that contained chemically induced pheochromocytomas. Owing to a highly significant ($p < 0.01$) difference among these chemicals, their scores could not be pooled. This significance was due entirely to TGP; therefore, this chemical was evaluated by itself. On the other hand, the staining grades of the other three chemicals that induced tumors were similar and were pooled for comparison with the spontaneously occurring pheochromocytomas. No significant differences existed in the grading score for CGA between spontaneously occurring benign and "malignant" tumors (Fig. 1C, 2C, respectively, and Table 6), although there was a suggestion that more cells in benign than "malignant" tumors stained for CGA. The CGA grading score from the three pooled chemicals was similar to that observed for the spontaneously occurring "malignant" and benign tumors and in the TGP group, the CGA grading was significantly ($p < 0.05$) reduced compared to these tumors.

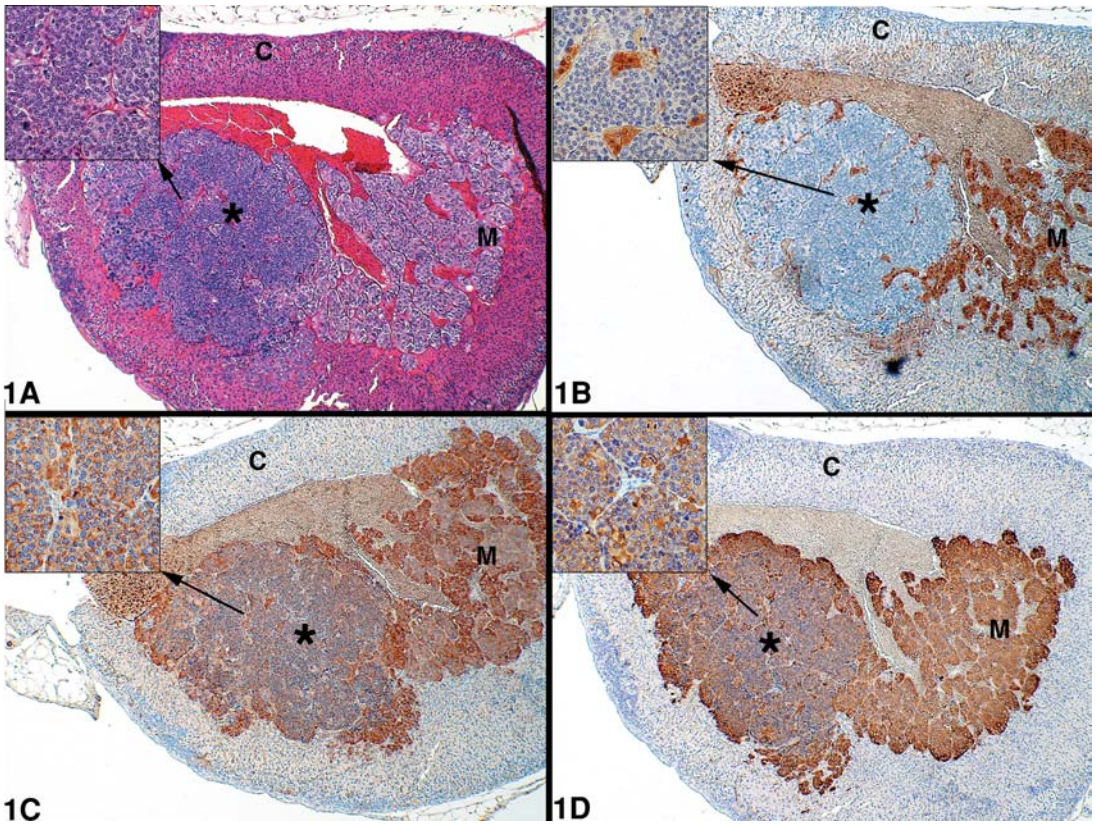

Fig. 1.

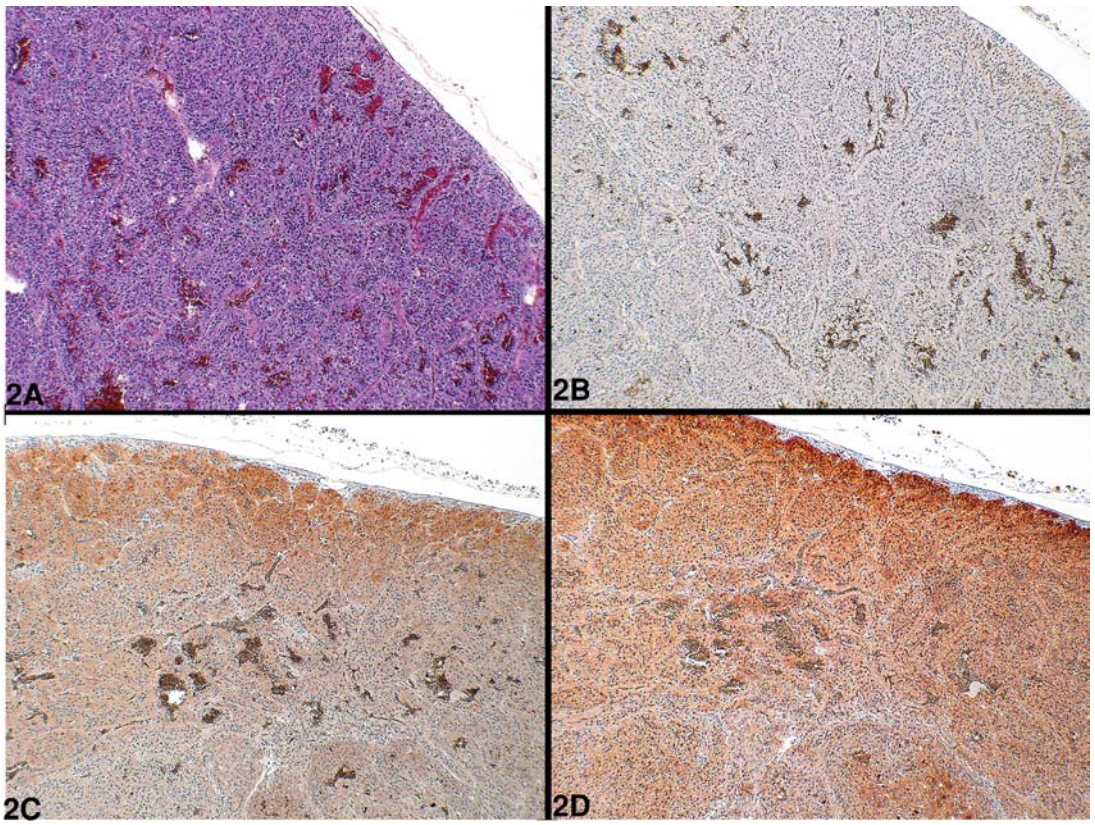

Fig. 2.

Fig. 1. Spontaneously occurring benign pheochromocytoma. Note the cortex (C) and the medulla (M). (×13.2; insets ×66.) **(A)*** Well-circumscribed nodule with pleomorphic cells and mitotic figure (inset). H&E. **(B)*** PNMT-minimal staining of nodule (grade 1). **(C)*** CGA-positive nodule (grade 4). **(D)*** TH-positive nodule (grade 4).

Fig. 2. Spontaneously occurring "malignant" pheochromocytoma. Note the obliteration of the cortex by expansile cords and clusters of neoplastic cells. (×13.2.) **(A)** Note numerous vascular spaces throughout tumor. H&E. **(B)** Minimal PNMT immunoreactivity (grade 1). **(C)** CGA-positive nodule (grade 4). **(D)** TH-positive nodule (grade 4).

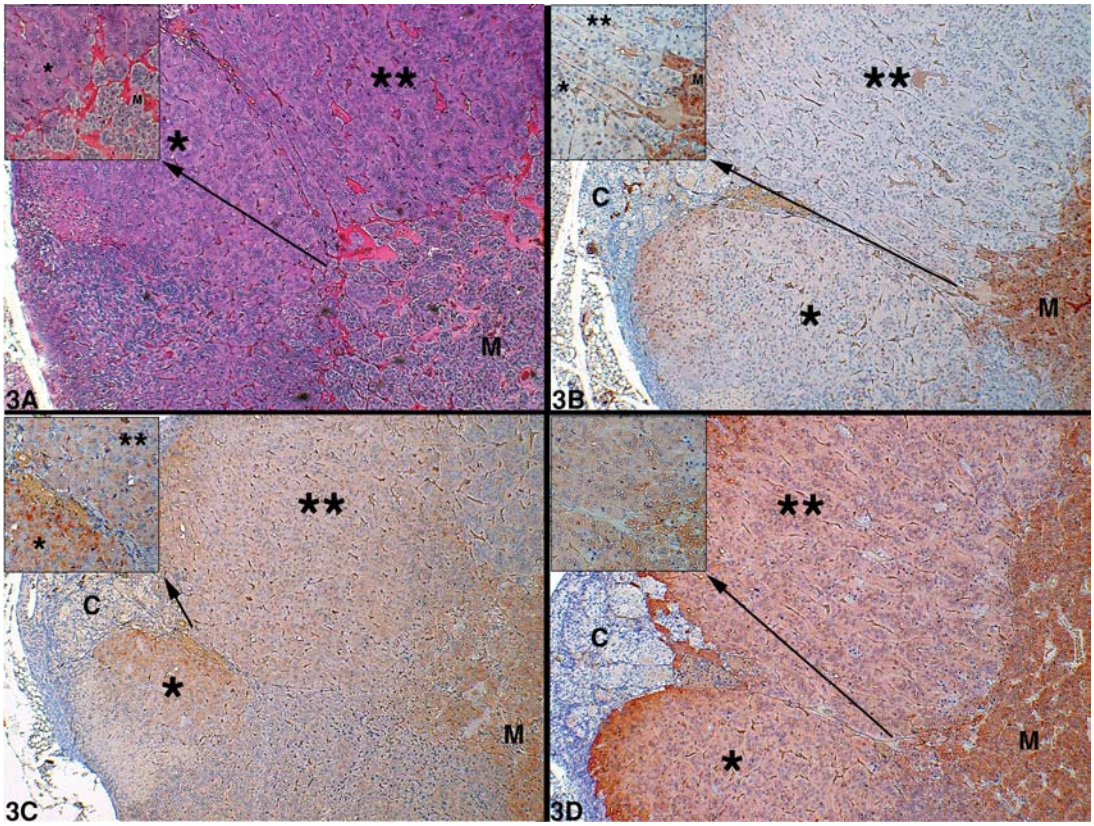

Fig. 3.

Fig. 3. Dowicide pentachlorophenol-induced benign pheochromocytoma. * and ** represent two well-circumscribed nodules; M = medulla. (×13.2; insets ×33.) **(A)** Note compression of cortex. H&E. **(B)** Note variability of PNMT expression between the two nodules. **(C)** Note slight variability of CGA expression between the two nodules. **(D)** TH-positive nodules (grade 4).

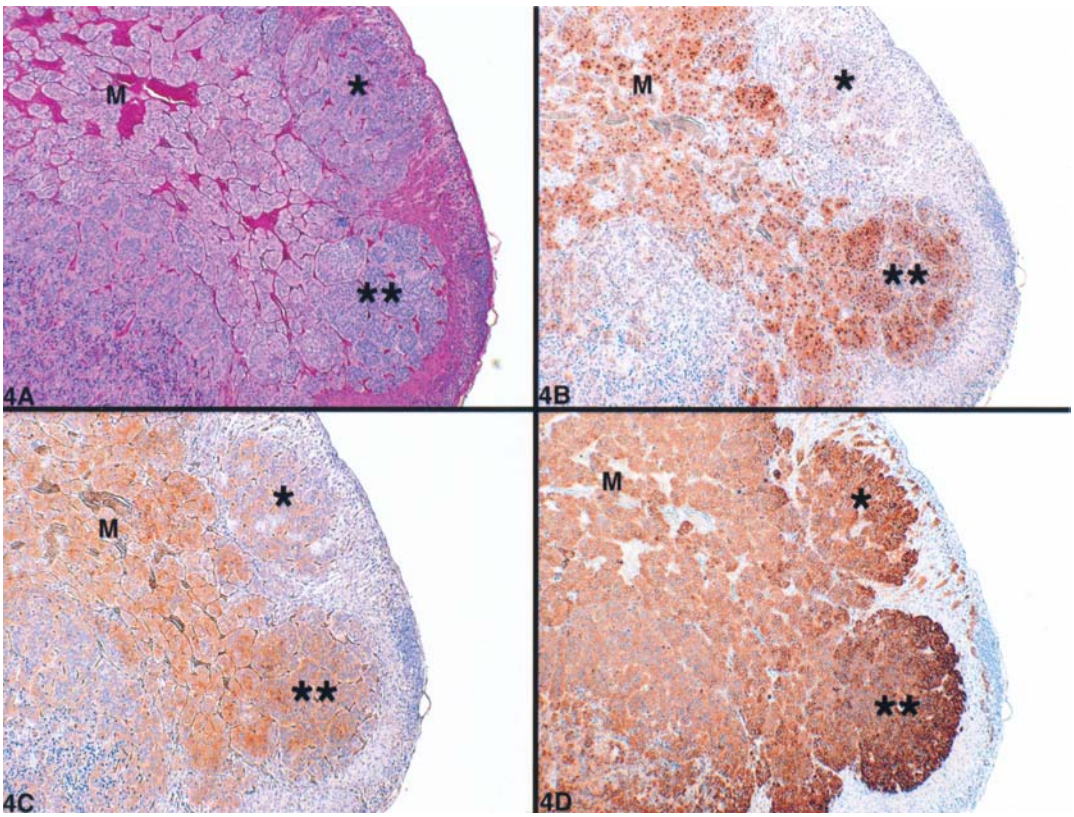

Fig. 4.

Fig. 4. Furan-induced benign pheochromocytoma. * and ** represent two well-circumscribed nodules; M = medulla. (All images ×13.2.) **(A)** Note compression of the cortex. H&E. **(B)** Note mild PNMT-positive nodule (*) and marked PNMT-positive nodule (**). **(C)** Variability in CGA staining between nodules. **(D)** TH-positive nodules (grade 4).

Table 5. Phenylethanolamine-N-methyltransferase (PNMT) Immunohistochemical Grading Summary in Mouse Pheochromocytomas

Grade	Spontaneous "malignant"	Spontaneous benign	Chemically induced benign
0	6	0	10
1	1	2	8
2	1	1	5
3	0	2	2
4	0	1	2
Median	0.00	2.50**	1.00*

*$p < 0.05$ vs spontaneous benign (Mann–Whitney *U* test).
**$p < 0.01$ vs spontaneous "malignant" (Mann–Whitney *U* test).

Table 6. Chromogranin A (CGA) Immunohistochemical Grading Summary in Mouse Pheochromocytomas

			Chemically induced benign	
Grade	Spontaneous "malignant"	Spontaneous benign	TGP**	Others
0	0	0	3	0
1	1	0	0	0
2	1	0	2	2
3	1	0	3	6
4	5	6	0	11
Median	4.00	4.00	2.00*	4.00

*$p < 0.05$ vs each of the other three groups (Mann–Whitney *U* test).
**TGP, Technical grade pentachlorophenol.

Discussion

The functional characteristics of chemically induced pheochromocytomas in mice have not been evaluated previously. Because these neoplasms have been diagnosed in B6C3F1 mice from several NTP studies, we felt that it would be of interest to compare the immunohistochemical features of these tumors with those from spontaneously occurring pheochromocytomas.

Spontaneously occurring mouse pheochromocytomas have been previously assessed immunohistochemically and shown to be variably immunoreactive for TH, PNMT, and CGA [17]. In the present studies, both chemically induced and spontaneously occurring tumors expressed these markers. Based on our scoring methods, TH was the enzyme that was most prevalent and consistently found in all of the pheochromocytomas. A distinction could not be made between the immunoreactivity of TH in the normal portions of the adrenal medulla and the pheochromocytomas. Although TH expression is not an absolute indicator of function, it has been shown to correlate well with the biosynthesis of catecholamines [9]. Our findings suggest that the spontaneously occurring and chemically induced pheochromocytomas function similarly to the normal adrenal medulla in general catecholamine synthesis.

Immunoreactivity for PNMT is a unique characteristic of mouse and most human pheochromocytomas [10,17]. In the present studies, there was variability in the percentage of positive-staining cells between the spontaneous "malignant," spontaneous benign, and chemically induced pheochro-

mocytomas (Table 5). We found that spontaneously occurring "malignant" tumors did not express PNMT, whereas most of the spontaneously occurring benign tumors did express this enzyme. These findings suggest that "malignant" pheochromocytomas do not synthesize epinephrine. Because there were no chemically induced "malignant" pheochromocytomas in this series, we could not determine whether this applies in all cases. Because we did not directly assay any of the tumors for catecholamines, we cannot be certain that immunoreactive PNMT is biochemically functional.

The lack of PNMT immunoreactivity in "malignant" pheochromocytomas could be related to any of several factors. One possibility is a less mature phenotype of the "malignant" cells, since PNMT is expressed later during embryogenesis than other catecholamine-synthesizing enzymes [1,17]. Another possibility is inability to maintain PNMT expression during "malignant" transformation. Alternatively, negative PNMT staining by "malignant" pheochromocytomas might be caused by mechanisms similar to those in a previously reported transgenic mouse model of multiple endocrine neoplasia type 2B (MEN-2B), in which it was speculated that the adrenal medullary tumor cells might be closely related to PNMT-negative chromaffin-like cells of the extraadrenal paraganglia that are located along the pelvic sympathetic nerves and in the sympathetic ganglia of postnatal mice [16].

Another possible explanation for the differences in immunohistochemical staining for PNMT between benign and "malignant" pheochromocytomas may lie in the clonal patterns of these tumors. A previous study in humans described the relationship between clonality, histological features, and cell kinetics in sporadic and MEN-2A adrenal medullary hyperplasias and pheochromocytomas [3]. In that study monoclonal proliferation was found in nodular adrenal medullary hyperplasia while polyclonal features were demonstrated in a subgroup of locally invasive pheochromocytomas. Although a criterion for malignancy in our study was local invasiveness (as opposed to distant metastasis being the most reliable criterion of malignancy in humans), it is possible that local invasiveness or tumor nodule size may be a factor in the PNMT staining of mouse pheochromocytomas. This theory might be applicable to the variability of PNMT expression between different neoplastic nodules within chemically induced benign pheochromocytomas. These results may indicate that nodules that expressed little or no PNMT could represent less-well-differentiated clones that have progressed further along the carcinogenic pathway or could be synthesizing catecholamines other than epinephrine.

CGA, located in the core of the secretory granules of most neuroendocrine cell types, has been widely used as a general neuroendocrine marker in histology and histopathology [15]. In our investigation, we found that overall CGA intensity was weak; however, the highest percentage of neoplastic cells expressing CGA was consistent among spontaneously occurring "malignant" and benign pheochromocytomas and three of the chemically induced tumors (Table 6). Interestingly, CGA immunoreactivity was reduced in pheochromocytomas in mice treated with TGP. The reason for this difference in CGA expression is unknown but may be related to the number of secretory granules that the neoplastic cells contained. It is also possible that TGP-induced pheochromocytomas processed CGA in such a way that they were not as sensitive as the other chemically induced pheochromocytomas to specific regions of CGA targeted by the

antibody. Unfortunately, tissue was not available for ultrastructural analysis in this retrospective study.

In a recent immunohistochemical study of rat pheochromocytomas, several markers, including TH and CGA, were evaluated [13]. Similarly to mice, TH was strongly and diffusely expressed both in benign and "malignant" pheochromocytomas confirming that catecholamine synthesis occurs at a very early stage of cell differentiation. Unlike most of the mouse pheochromocytomas from our study, CGA expression in the rats varied among different areas of the tumors. This may be the result of differences in the cellular content of secretory granules, possibly the grade of differentiation, maturation and functionality of the neoplastic cells, or potential differences in staining sensitivity between species.

The findings from this study confirm that immunohistochemical analysis of pheochromocytomas in mice provides insight into the functional capabilities of these tumors. Although we cannot definitively state that the biological behavior of spontaneously occurring (either benign or "malignant") or chemically induced pheochromocytomas can be predicted using the described methods, comparisons concerning catecholamine synthesis can be made. Furthermore, our findings may be beneficial in providing potential information for an animal model for human pheochromocytomas.

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