Life Sciences, Vol. 59, No. 19, pp. 1659-1665, 1996
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0024-3205/96 $15.00 + .00

ELSEVIER

PII S0024-3205(96)00497-3

TYROSINE HYDROXYLASE GENE EXPRESSION IN VARYING FORMS OF HUMAN PHEOCHROMOCYTOMA

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(Received in final form August 30, 1996)

Summary

We performed a comparative study of catecholamine content, tyrosine hydroxylase (TH) activity, and TH mRNA levels in normal human adrenals and various clinical forms of human pheochromocytoma. We studied sporadic, benign intra-adrenal chromaffin tumors and other non-malignant intra-adrenal tumors associated with multiple endocrine neoplasia type 2B (MEN 2B) and von Hippel-Lindau disease along with one extra-adrenal malignant pheochromocytoma. Our findings suggest substantial differences in TH transcriptional rates or the stability of TH mRNA or both may contribute to altered TH expression in human chromaffin cells associated with "normal" adrenal tissues and various forms of pheochromocytoma and distinctive patterns of expression in the different settings in which these tumors arise.

Key Words: pheochromocytomas, tyrosine hydroxylase, gene expression, catecholamines, von Hippel-Lindau disease, MEN 2B syndrome

Pheochromocytomas are uncommon tumors derived from neuroectodermal chromaffin tissue (1,2). While their presence may be unrecognized clinically, production of catecholamines by these neoplasms typically results in a variety of severe cardiovascular manifestations, especially hypertension, often in a paroxysmal pattern. The rarity of pheochromocytomas is indicated by their occurrence in fewer than 0.1% of patients with hypertension (2-4), with an incidence of between 0.4 - 8.0 cases per million individuals per year in various population surveys (5). Despite their uncommonness, however, these tumors are of importance clinically because they are potentially lethal, yet are curable in over 90% of cases when recognized and removed (2,6). While pheochromocytomas typically arise within the adrenal medulla, approximately 10% occur in extra-adrenal sites (1,2,4,7).

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Pheochromocytomas secrete norepinephrine and/or epinephrine at a high rate in an often unregulated fashion (8-15). The majority of these tumors, however, do not secrete substantial amounts of dopamine (13). Most pheochromocytomas are also characterized by a high tissue catecholamine content. Tyrosine Hydroxylase (TH), the rate limiting enzyme in catecholamine formation (16-17), is highly active in pheochromocytomas (10,13,18,19) and in PC-12 cultures (20), a pheochromocytoma cell line derived from the rat. Findings in PC-12 cells suggest that the increase in catecholamine biosynthesis in these tumors may be a direct result of the over-expression of TH. However, there have been few direct assessments of TH mRNA levels in human pheochromocytomas or correlations of this parameter with TH activity or catecholamine content. One recent report found over expression of TH, dopamine ß-hydroxylase and aromatic l- amino acid decarboxylase. (21).

Sporadically-occuring pheochromocytomas are the most common form of human pheochromocytoma and may range in size from a few grams to more than a kilogram. Virtually all patients with these tumors have elevated urinary metanephrines (catecholamine metabolites) but only approximately half of such individuals show any detectable form of hypertension. Unlike the normal adrenal medulla, whose primary catecholamine secretory product is epinephrine (19), sporadic pheochromocytomas usually produce predominantly norepinephrine (22,23).

Other types of human pheochromocytomas with extremely low clinical incidence include malignant chromaffin cell tumors of intra and extra-adrenal origin representing 6 - 10% of such tumors in various series as well as pheochromocytomas associated with MEN-2 syndromes (A and B) and von Hippel Lindau disease occuring even less frequently (2). MEN-2 syndromes are characterized by multiple endocrine tumors. Patients with MEN 2B typically have not only epinephrine-secreting pheochromocytoma (22) but also medullary thyroid carcinoma and mucosal neuromas. This disorder is thought to be due to germline mutations in the RET tyrosine kinase proto-oncogene (24, see also review 25). For von Hippel Lindau disease, the occurrence of pheochromocytoma is typically secondary to retinal and cerebellar hemangioblastomatosis, and the clinical symptomatology characteristic of inappropriate catecholamine secretion is often absent. A defective tumor suppressor gene has been reported in this syndrome at the 3 p25-p26 region of the human genome (26). The majority of mutations observed in this genetic disorder appear to be deletions. In a previous study, we observed marked differences in catecholamine levels in chromaffin cell tumors from these rare syndromes vs. those of sporadic nature (23).

To determine whether differences in tissue catecholamine content in various forms of human pheochromocytoma are directly associated with TH activities and the abundance of TH mRNA transcripts, we have measured these parameters in tumors from patients with sporadic, Von Hippel-Lindau, MEN 2B, and malignant extra-adrenal pheochromocytoma as well as in normal adrenal tissues and "morphologically 'normal' remnant adrenal tissues" adjacent to these various tumors.

Materials and Methods

Patients

Pheochromocytoma was suspected preoperatively in the 10 patients studied (6 men, 4 women) based on accepted clinical and laboratory criteria, including elevated urinary catecholamine and metabolite measurements and demonstration of a mass by appropriate imaging procedure. The diagnoses of multiple endocrine neoplasia and Von-Hippel Lindau disease were established clinically. Patients were operated on at the Universtiy of Miami/Jackson Memorial Medical Center or at nearby affiliated hospitals. The diagnosis of pheochromocytoma was confirmed and the benign or malignant nature of the tumor established by pathologic examination of the resected specimen.

Tissue Processing

Human adrenal glands and pheochromocytomas were obtained directly from the operating suites or surgical pathology laboratories of neighboring medical centers placed in cold saline immediately after removal and frozen on dry ice within approximately an hour after removal. All tissue samples were stored at -70° to -80°C before assay. For TH and TH mRNA analyses, representative sections of tissue were weighed and homogenized in 100 μl of buffer consisting of 50

mM Tris-EDTA, 0.2%, Triton X-100, pH 7.4. Twenty-five μl quantities of homogenate were removed for determinations of protein content (27), TH mRNA (28), and TH enzyme activity (28). For catecholamine measurements, tissues were homogenized directly in 0.1 M perchloric acid (PCA).

Reagents and biochemicals

L-[^{14}C]-tyrosine and [α-^{32}P] d ATP were obtained from Amersham (Arlington Heights, IL) and dimethyl-5,6,7,8-tetrahydropterin hydrochloride (6-MPH$_4$) was purchased from Calbiochem (San Diego, CA). "RNAzol" was obtained from Biotecx (Friendswood, TX). Other materials and reagents were obtained from the Sigma Chemical Company (St. Louis, MO).

Analyses of tyrosine hydroxylase activity

TH activity was measured by a coupled decarboxylase technique. This assay is based on trapping radiolabeled CO_2 evolved through the conversion of [L-^{14}C]-tyrosine into dopamine (29). In summary, each 10 μl tissue homogenate was diluted to 100 μl with homogenizing buffer. The diluted sample was then passed over a Sephadex G50 column to remove endogenous catecholamines. Portions of the eluate and buffer (6 μl) were assayed in duplicate as blanks in a final volume of 10 μl; the saturating concentrations of the cofactor 6-MPH$_4$ was 1 mM. The final concentration of tyrosine was 100 μM and the final pH of the reaction mixture was 6.5. The incubation assay also contained 33.0 units/ml of catalase, 10% Triton X-100, 1 M n-morpholino ethanol sulfonic acid (MES), 20 mM dithiothreotol (DTT), and 40 mM ascorbic acid.

Samples were first incubated at 37°C for 15 minutes and the reaction stopped by placing assay tubes in an iced-water bath and adding 10 μl of a cyanide mixture consisting of 33.34 mM potassium ferricyanide and 20 mM p-chloromercuriphenyl sulfonic acid. Assay vials were vortexed after connection to CO_2 trapping tubes (50 ul of methylbenzethonium hydroxide) and immersed in a water bath at 60°C for 10 minutes. Ten percent trichloroacetic acid was then injected into the vials. Incubation was continued for one hour at 37°C. The methylbenzethonium hydroxide containing tubes were then placed in counting vials with 10 ml of scintillation fluid and radioactivity quantified by scintillation spectrometry.

Assessment of TH mRNA levels

The concentration of total cellular RNA from adrenals was determined by extraction using a modification of our previously published method (28). Sonicated tissue (75 µl homogenate) was extracted with "RNAzolB" (a mixture of phenol, guanidinium thiocyanate, Biotecx, Friendswood, TX) (30). The integrity of the isolated RNA was verified using agarose (1%) gel electrophoresis in comparison with 18S and 28S RNA standards (Sigma, St. Louis, MO).

The pBR322 recombinant plasmid containing TH.36 cDNA (kindly supplied by Dr. Karen O'Malley; Washington University, St. Louis, Mo.) was grown in E. coli and plasmid DNA isolated by standard procedures. The cDNA was excised from the plasmid DNA with Eco RI and purified by electrophoresis on a 1% low-melting-temperature (LMT) agarose gel. The cDNA was eluted from the LMT gel with equilibrated phenol and labeled by nick translation using [α-^{32}P]dATP (3,000 Ci/mmol) to a specific activity of 8 x 10^8 cpm/μg, and 1 x 10^6 cpm/ml was used in the hybridization medium. The labeled probe was purified using Elu-tips syringe columns and heat denatured by boiling for 10 min before use.

For measurement of TH mRNA levels by slot blot analysis, equal concentrations of serially diluted RNA samples were immobilized on Gene Screen nylon membranes using a Bio-Rad slot blot apparatus. RNA isolated from the cerebral cortex was used as a negative control. The filters were heated to 80°C for 2-4 h and prehybridized using 25 mM potassium phosphate, 5X SSC, 5X Denhardt's solution, 50 μg/ml denatured salmon testes DNA, and 50% formamide. After incubation for 14-16 hours at 42°C, the filter was hybridized with a [^{32}P] nick translated, full length TH.36 cDNA probe. The filter was then washed and exposed to Kodak (Rochester, NY) X-AR film for 72

hours. Autoradiograms were scanned using a Bio-Rad Model 620 densitometer and optical density per μg of total cellular RNA was calculated from the linear portion of the curve.

Although tissues were collected in a consistent manner, it remains possible that THmRNA levels may have been affected in some instances by degredation prior to freezing.

Catecholamine analyses

Catecholamines were extracted with 0.1 M perchloric acid by the method of Watson (31) and adsorbed onto aluminum oxide after the addition of 3,4-dihydroxybenzylamine (DHBA) as an internal standard. Dopamine, norepinephrine, and epinephrine were quantitated by HPLC using minor modifications of the techniques described by Mayer and Shoup (32). The mobile phase, with pH adjusted to 3.45 consisted of 14.2 g/l monochloroacetic acid, 4.7 g/l sodium hydroxide, 0.75 g/l EDTA, and 2.5 ml/l of a 4% (w/v) solution of sodium octyl sulfate. This mixture was passed through a 0.45 μm Millipore filter before use. The HPLC apparatus consisted of a ConstaMetric pump (Model III, LDC/Milton Roy, Riviera Beach, FL), a 15 cm Microsorb C18 bonded-phase column (Model 80-125, Rainin Instrument Co., Woburn MA) and an amperometeric detector (Model LC-4B, Bioanalytical Systems, West Lafayette, IN) equipped with Ag/AgCl and glassy-carbon electrodes. Individual values for dopamine, norepinephrine, and epinephrine have been added together and reported here as total catecholamines.

Results and Discussion

Adrenal Tumors

The six sporadic pheochromocytomas studied ranged in weight from 6 to 210 g with an average weight of 81 g (Table 1). We found no correlation between tumor weight and catecholamine content on a per gram tissue basis. Similarly, tumor weight did not correlate with cellular TH mRNA levels or TH enzyme activity; however, TH mRNA did correspond in most instances with both TH activity ($r = 0.8762$; $p = 0.05$) for 4 of 6 tumors studied and total catecholamine content ($r = 0.7584$; $p = 0.08$). Additionally TH activity also correlated with catecholamine content ($r = 0.8328$; $p < 0.04$).

One sporadic tumor (#5), however, was a clear exception to this general finding. In this case, TH mRNA levels were extremely high, whereas TH activity was below the detection threshold and the total catecholamine content was negligible (0.06 mg/g vs. an average of 2.41 mg/g of tissue for the six sporadic pheochromocytomas studied). This finding suggests a mutation in the TH gene or TH regulatory elements of this tumor, resulting most likely in a diminished or untranslated message or, alternately, a relatively inactive TH translation product. If the TH gene were actively feedback inhibited by dopamine or other catecholamine products (norepinephrine, epinephrine or their metabolites) or both, then one might expect the TH mRNA results observed. Low catecholamine levels in this circumstance would be expected to activate the TH gene, leading ultimately to high rates of TH mRNA transcription yet low TH activity and consequently, continued low tissue catecholamine levels. The ratio of TH activity to catecholamine content in this tumor approached zero whereas the other tumors displayed TH/CA ratios ranging between 2 and 5 with one outlying value of 23 (Table 1).

The single 16 g MEN 2B pheochromocytoma studied contained the highest TH mRNA levels and also the highest TH activity relative to all other tissues and tumors examined. Catecholamine content was also high in the MEN 2B syndrome pheochromocytoma relative to tumors from von-Hippel Lindau disease and malignant extra-adrenal pheochromocytoma (1.87 mg/g of tissue vs 0.22 and 0.01 mg/g of tissue, respectively); however, catecholamines were not elevated in this tumor relative to sporadic intra-adrenal pheochromocytomas.

By examining the ratios of TH activity to catecholamine content, a distinct pattern is apparent between these various tissues. In MEN 2B syndrome this ratio was 33 (N=1), whereas, in sporadic

intra-adrenal tumors it averaged 6 ± 9 (N=6). In von Hippel-Lindau disease it averaged 14 ± 8 (N=2), whereas in malignant extra-adrenal pheochromocytoma it measured 0 (N=1).

Although only one case of MEN 2B pheochromocytoma was studied, the findings obtained imply an increased rate of TH mRNA transcription and higher rates of catecholamine release and turnover, a combination of traits likely to suggest more serious clinical complications. In other studies where catecholamine content was examined in a large number of these and other types of pheochromocytomas the epinephrine (E) to norepinephrine (NE) ratio was found to be substantially higher in MEN 2B tumors than in sporadic pheochromocytomas (22,23). These findings, would be expected to increase a tendency toward hypertension.

The two pheochromocytomas studied that were associated with von Hippel-Lindau disease (Table 1) both had low THmRNA levels, TH activities, and catecholamine contents. These data suggest that this form of tumor may be less clinically threatening from a hypertensive perspective than most sporadic pheochromocytomas and those adrenal medullary tumors associated with MEN 2B syndrome.

The single malignant extra-adrenal pheochromocytoma (of 1,030 g) examined had extremely low levels of THmRNA, TH activity, and catecholamine contents compared to all other pheochromocytomas studied and also compared to other normal or remnant normal adrenal tissues adjacent to pheochromocytomas or other types of adrenal tumors. This finding is not surprising considering the highly undifferentiated nature of a malignant tumor and its isolation from adrenal cortical steroid influences because of its extra-adrenal location.

Normal adrenal glands and remnant "normal" tissues from adrenal tumors

All forms of normal human adrenal medullary tissue studied, including those adjacent to various adrenal tumors (Table 2) showed nondetectable THmRNA levels and very low catecholamine contents compared to all pheochromocytomas examined except for the single case of a malignant extra-adrenal tumor (described above). TH activity, however, was readily detected in these normal tissues. Although the activities of this enzyme were somewhat lower in normal tissues than in those of sporadic pheochromocytomas, they were similar in magnitude to activities in the two tumors associated with von Hippel-Lindau disease but were substantially higher than observations in the malignant extra-adrenal pheochromocytoma where TH was undetectable.

Perhaps the most intriguing comparisons in these data are found between normal tissues and the pheochromocytoma from MEN 2B syndrome where TH activity was extremely high. Interestingly, TH activity was also strongly elevated in the so-called normal adrenal remnant tissue from the patient with the MEN 2B associated pheochromocytoma, although TH mRNA levels and total catecholamines in this tissue were again, as in other normal tissues, low or nondetectable.

However, since the number of MEN 2b, von Hippel Lindau, and malignant extraadrenal tumors that were analyzed was very small, it may be less useful to stratify the TH/CA ratio by individual tumor type than to compare this ratio in pheochromocytomas overall (i.e., tumors without distinction to their associated syndrome) relative to "normal" adrenal glands or tissues. Analyzed in this way, TH/CA ratios were > 10 in only 3 of 10 pheochromocytomas studied but they were > 10 in 4 of 5 normal or remnant normal adrenal glands.This finding suggests that small increases in TH activity can result in much larger increases in catechalomine levels.

Conclusions

These studies show dramatic differences in not only total catecholamine content but also TH activity and TH mRNA levels between normal adrenal tissues and different forms of human pheochromocytoma. These findings suggest substantial differences in TH transcriptional rates or stability of TH mRNA or both, contributing to altered TH expression in human chromaffin cells associated with normal adrenal tissues and various forms of pheochromocytoma and distinctive patterns of expression in the different settings in which these tumors arise.

Acknowledgments

The authors thank their surgical colleagues, Dr. George Irvin and Dr. Norman Block for their help in procuring adrenal tissues and tumors and Ming-Cheng Liu and Michael Matheny for their assistance in TH mRNA and TH enzyme activity assays. We regret to announce the passing away of our co-author, Dr. Andres Carballeira, before the publication of this manuscript. We dedicate this paper to his memory. These studies were supported by the U.S. Department of Veterans Affairs.

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