PITUITARY MAMMOSOMATOTROPH ADENOMAS DEVELOP IN OLD MICE TRANSGENIC FOR GROWTH HORMONE-RELEASING HORMONE

SYLVIA L. ASA, KALMAN KOVACS, LUCIA STEFANEANU, EVA HORVATH, NILS BILLESTRUP, CONSUELO GONZALEZ-MANCHON AND WYLIE VALE

Department of Pathology, St. Michael's Hospital, University of Toronto, Toronto, Ontario M5B 1W8 Canada, Hagedorn Research Laboratory, Gentofte, Denmark DK-2820 and The Salk Institute, La Jolla CA 92037 U.S.A.

Abstract. It has been shown that mice transgenic for human growth hormone-releasing hormone (GRH) develop hyperplasia of pituitary somatotrophs and mammosomatotrophs, cells capable of producing both growth hormone and prolactin, by 8 months of age. We now report for the first time that old GRH-transgenic mice, 16 to 24 months of age, develop pituitary mammosomatotroph adenomas. These findings provide conclusive evidence that protracted stimulation of secretory activity can cause proliferation, hyperplasia and adenoma of adenohypophysial cells.

Acromegaly and gigantism are due to excessive growth hormone (GH) production by the pituitary; the most frequent pathology underlying these disorders is a pituitary adenoma producing GH (1). The pathogenesis of pituitary tumors is not known. It has been suggested that they may be dependent on increased stimulation or decreased inhibition by the hypothalamic peptides that physiologically regulate hormone secretion of adenohypophysial cells (1,2). In the case of GH-producing adenomas, hormone secretion can be stimulated by GH-releasing hormone (GRH) and suppressed by somatostatin (3). A pathogenetic role for GRH in the formation of GH cell adenoma has been suggested (1,2,4,5), however, this hypothesis was not proved so far.

Prolonged GRH excess occurs rarely in man and is due to GRH-secreting extrapituitary tumors (6); this disorder offers an experiment of nature to study the effects of chronic GRH stimulation on the pituitary. Overproduction of GRH by the tumor increases pituitary GH release, giving rise to elevated blood GH levels and the development of acromegaly or gigantism (6). The pituitaries of patients bearing extra-pituitary GRH-secreting tumors show diffuse or nodular hyperplasia of GH-containing cells (6); one patient with a pancreatic GRH-producing tumor had a pituitary GH-containing adenoma (6). In addition, GRH-containing hypothalamic gangliocytomas have been associated with pituitary somatotroph adenoma (7), however, the role of GRH in the formation of pituitary tumors remained uncertain.

The development of transgenic animals allows insertion of hormone genes and investigation of the protracted effects of those hormones. Mice transgenic for GRH are known to have increased body weight and size as well as hyperplasia of the target of GRH, the pituitary (8,9). We report the occurrence of GH- and prolactin-producing pituitary adenomas in old mice transgenic for human GRH (hGRH). These results support the suggestion that sustained GRH excess is involved in pituitary tumorigenesis.

0037-9727/90/1933-0232$2.00/0

Materials and Methods

The hGRH/mouse metallothionein I/SV40 small t fusion gene was used to develop transgenic mice (9). A 713 base pair fragment of the mouse MT-1 promoter, containing elements responsible for metal induction and transcription initiation, was fused to 220 base pairs of the hGRH gene, encoding the NH_2-terminal 31 amino acid signal peptide and the 40 amino acid form of hGRH. The polyadenylation signal was provided by fusion to an 847 base pair fragment of the SV40 virus small t poly A; this portion of the gene is involved only in polyadenylation and is not translated. Fertilized zygotes of the B6D2F1 hybrid strain of mice, produced by mating C57BL/6 females and DBA/2 males, were microinjected with purified DNA fragments. Transgenic pups in a litter were identified at time of weaning; tail DNA was analyzed for the presence of hGRH by a DNA dot blotting method. From 6 weeks of age, the animals were maintained on water containing 25 nM $ZnSO_4$ and laboratory chow ad libitum. Blood was collected from the tails of 8 week old animals to measure serum levels of mouse GH and hGRH by radioimmunoassay (9). Transgenic mice and age- and sex-matched controls were sacrificed by decapitation. At autopsy, the pituitaries were removed and weighed; the other organs were carefully inspected. For light microscopy, portions of each pituitary were fixed in formalin and embedded in paraffin; sections were stained with hematoxylin and eosin, the Gordon-Sweet silver method to demonstrate the reticulin fiber network, and immunocytochemical stains to localize adenohypophysial hormones using the avidin-biotin-peroxidase complex. Primary antisera were donated by Dr. A.F. Parlow at the National Pituitary Agency (NIADDK, Bethesda, MD) (1,6,7,9). For transmission electron microscopy, pieces of tissue were fixed in glutaraldehyde, postfixed in osmium tetroxide and embedded in epoxy resin. Ultrastructural immunocytochemistry utilized the double immunogold technique (1,9).

Results

All animals identified as transgenic for hGRH had significantly increased body weight and elevated blood levels of GH and hGRH. The pituitary glands were markedly enlarged. Microscopic studies of the pituitaries of six trans-

genic animals at 8 months of age and one 13 month old mouse showed hyperplasia of somatotrophs and of bihormonal GH- and prolactin-containing cells called mammosomatotrophs; the morphologic findings in these mice are reported elsewhere (9). All 6 transgenic animals that were older than 13 months when sacrificed had evidence of pituitary adenoma. Four had grossly enlarged pituitaries which were adherent to the brain; these were not examined microscopically. Two animals aged 16 and 24 months had enlarged and hemorrhagic pituitary glands which showed diffuse somatotroph and mammosomatotroph hyperplasia similar to that seen in younger animals (9); in addition, each animal had a discrete tumor (Fig. 1). Occasional binucleate cells were seen and mitotic figures were readily identified in the adenomas. The Gordon-Sweet silver stain documented the presence of a distorted reticulin network at the periphery of the adenomas with absence of reticulin fibers within the tumors. The majority of tumor cells showed intense immunoreactivity for GH; numerous tumor cells exhibited prolactin positivity as well. Other adenohypophysial hormones were detected in cells within the hyperplastic areas but not within the tumors; the cells containing these hormones were of normal size and shape and had usual ultrastructural features. Electron microscopy revealed that the tumors were composed of cells with features of mammosomatotrophs (1,9) (Fig. 2a). The cytoplasm contained moderate numbers of pleomorphic secretory granules measuring up to 1000 nm; misplaced exocytoses of granular material were conspicuous. The immunogold technique documented the presence of GH and prolactin within the secretory granules of most adenoma cells and in granule extrusions; both hormones were often colocalized in the same secretory granules (Fig. 2b). A few adenoma cells were labelled only for GH.

Nontransgenic littermates served as controls in this study; at 8 months (n=7), 12 months (n=2), 15 months (n=3) and 2 years (n=3), there was no evidence of adenohypophysial hyperplasia or neoplasia in the pituitaries of those mice.

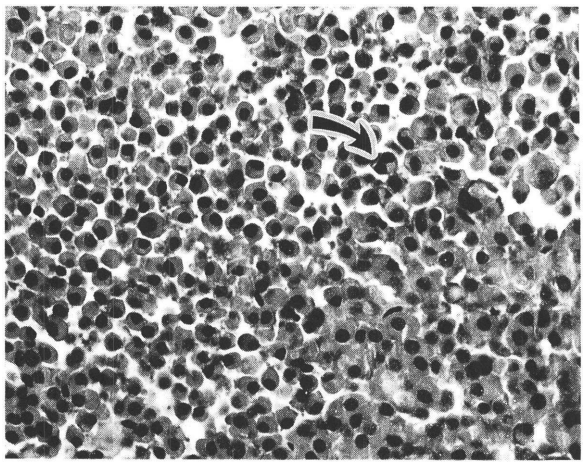

Figure 1. Pituitary adenoma of 16 month old mouse transgenic for hGRH. Acinar architecture is disrupted and a binucleate cell is seen (arrow). Hematoxylin-eosin stain; original mag. × 64.

Discussion

Although previous studies have shown that chronic stimulation of rat adenohypophysial cells by GRH *in vitro* causes somatotroph proliferation as measured by [^{3}H]thymidine uptake (10) and that GRH stimulates the expression of the *c-fos* proto-oncogene in cultured somatotrophs (11), there was no direct proof that protracted GRH excess can be implicated in adenoma formation. Pituitary adenomas are not known to occur in the parent strains of the hybrid mouse strain studied (Russell JD, Simonsen Laboratories Inc., Gilroy CA; personal communication) and control animals had no evidence of adenohypophysial hyperplasia or neoplasia. In contrast, the pituitaries of 4 transgenic animals, although not examined microscopically, were adherent to brain, and were grossly consistent with a diagnosis of adenoma. The morphologic criteria of adenoma (1) were

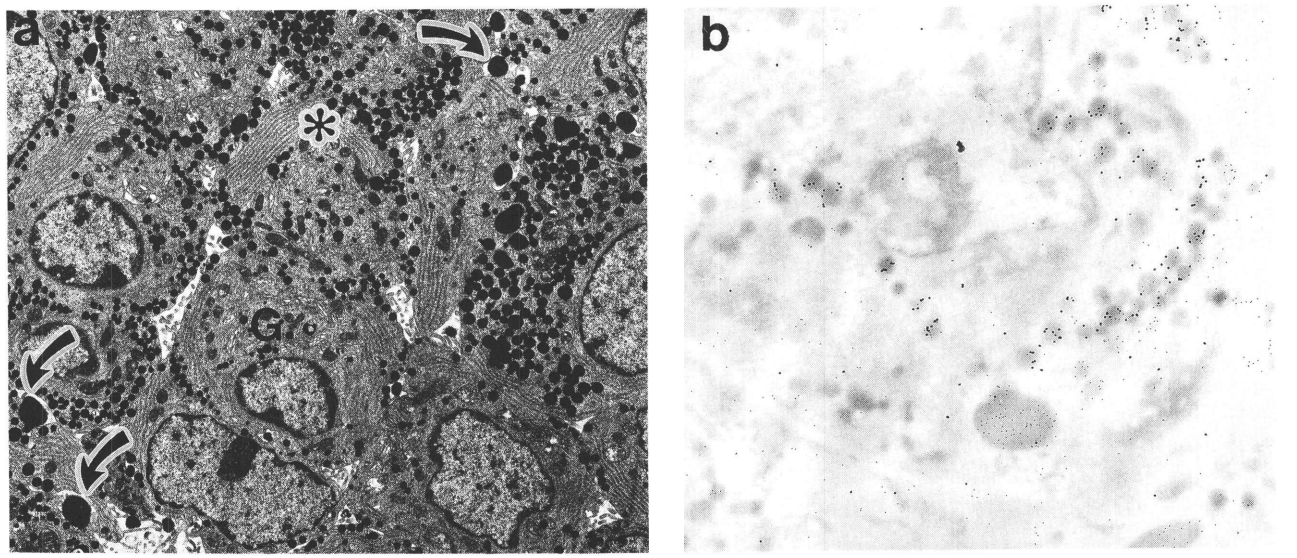

Figure 2. Electron microscopy of pituitary adenoma in 2 year old mouse transgenic for hGRH. (a) Tumor cells have well developed endoplasmic reticulum (*) and Golgi complexes (G) and numerous large, pleomorphic secretory granules; extrusion of secretory material is conspicuous (arrows). Mag. × 4100. (b) The immunogold technique conforms the presence of both GH and prolactin in adenoma cells. Almost all secretory granules contain label for GH (15 nm gold particles), several contain only label for prolactin (28 nm gold particles) and in many, both hormones colocalize in the same secretory granule. Mag. × 7600.

fulfilled unequivocally in both pituitaries examined histologically: there was a discrete, well demarcated nodule compressing adjacent tissue; the reticulin pattern was disrupted; the cellular composition was distinctively different from the remainder of the gland. Thus it is reasonable to assume that the prolonged exposure to GRH in these old transgenic mice plays a major role in the development of pituitary mammosomatotroph adenoma. Moreover, this represents the first time that introduction of a single hormone gene into the genome of an animal has been shown to induce tumors.

More work is needed to clarify the role of GRH in the pathogenesis of pituitary adenomas. According to the multistep theory of carcinogenesis (12), GRH can act as an initiator or a promoter of neoplastic growth. In these transgenic mice, GRH may induce tumors by increasing the population of proliferating cells which are susceptible to other oncogenic factors or spontaneous transformation. This would explain the occurence of solitary neoplasms in older animals in association with hyperplasia similar to that seen in younger animals. Cell proliferation may be mediated by other factors, such as various growth factors which are known to exist in the anterior pituitary (13,14). Alternatively, it is possible that cell transformation may be the direct effect of longterm GRH stimulation.

Hormonal stimulation may play a role in the etiology of several neoplasms. There is evidence of hormone-dependent growth of carcinomas arising in breast, endometrium and prostate (15-17); among endocrine organs, thyroid tumors may be stimulated by thyrotropin (18), some tumors of adrenal cortex are thought to be dependent on ACTH stimulation (19) and in rodents, administration of estrogens results in pituitary lactotroph adenoma (20). Endocrine insufficiency has been implicated in tumorigenesis of thyroid, adrenals and gonads (18,19,21,22); the chronic compensatory hormonal stimulation by pituitary regulatory hormones is thought to play a role in the growth of these neoplasms. Similarly, loss of feedback inhibition may account for some parathyroid adenomas in tertiary hyperparathyroidism (23) and some pituitary adenomas composed of corticotrophs thyrotrophs or gonadotrophs (1); the complex regulation of these cells raises the possibility that their proliferation is also modulated by sustained hormonal stimulation.

The neoplasms in mice transgenic for GRH subjected to prolonged hormonal stimulation arose in association with diffuse hyperplasia. While human pituitary adenomas are more commonly solitary tumors which are neither preceeded by nor associated with hyperplasia, the oncogenic potential of GRH shown by this model suggests that GRH and possibly other stimulating peptides may, under certain circumstances, play a role in adenoma formation in humans. Further studies of models such as these transgenic mice may clarify the role of various oncogenic agents and hormonal stimulation in endocrine tumorigenesis.

Acknowledgements

J. Price, the Salk Institute, Biotechnology/Industrial Associates, Inc., San Diego CA, prepared the transgenic animals. This work was supported by Grants of the Medical Research Council of Canada and the National Institutes of Health, U.S.A. The research was conducted in part by the Clayton Foundation for Research, California Division. W.V. is a senior Clayton Foundation investigator.

References

1. Kovacs K, Horvath E. Tumors of the Pituitary Gland. Atlas of Tumor Pathology, Fascicle 21, Second Series. Washington DC: Armed Forces Institute of Pathology, 1986.
2. Asa SL, Kovacs K. Development and proliferation of adenohypophysial cells. In: Falkmer S, Häkanson R, Sundler F, Eds. Evolution and Tumour Pathology of the Neuroendocrine System. Amsterdam: Elsevier, pp399-425, 1984.
3. Reichlin S. Control of GH secretion: an overview. In: Lüdecke DK, Tolis G, Eds. Growth Hormone, Growth Factors and Acromegaly. New York: Raven Press, pp1-11, 1987.
4. Melmed S, Braunstein GD, Horvath E, Ezrin C, Kovacs K. Pathophysiology of acromegaly. Endocr Rev 4:271-290, 1983.
5. Molitch ME. Pathogenesis of pituitary tumors. Endocrinol Metab Clin 16:503-507, 1987.
6. Sano T, Asa SL, Kovacs K. Growth hormone (GRH)-producing tumors: clinical, biochemical and morphologic manifestations. Endocr Rev 9:357-373, 1988.
7. Asa SL, Scheithauer BW, Bilbao JM, Horvath E, Ryan N, Kovacs K, Randall RV, Laws ER Jr, Singer W, Linfoot JA, Thorner MO, Vale W. A case for hypothalamic acromegaly: a clinicopathological study of six patients with hypothalamic gangliocytomas producing growth hormone releasing factor. J Clin Endocrinol Metab 58:796-803, 1984.
8. Mayo KE, Hammer RE, Swanson LW, Brinster RL, Rosenfeld MG, Evans RM. Dramatic pituitary hyperplasia in transgenic mice expressing a human growth hormone-releasing factor gene. Mol Endocrinol 2:606-612, 1988.
9. Stefaneanu L, Kovacs K, Horvath E, Asa SL, Losinski NE, Billestrup N, Price J, Vale W. Adenohypophysial changes in mice transgenic for human growth hormone-releasing factor (hGRF): a histologic, immunocytochemical and electron microscopic investigation. Endocrinology, 1989, in press.
10. Billestrup N, Swanson LW, Vale W. Growth hormone releasing factor stimulates proliferation of somatotrophs in vitro. Proc Natl Acad Sci USA 83:6854-6857, 1986.
11. Billestrup N, Mitchell RL, Vale W, Verma IM. Growth hormone-releasing factor induces c-fos expression in cultured primary pituitary cells. Mol Endocrinol 1:300-303, 1987.
12. Farber E. Chemical carcinogenesis. New Engl J Med 305:1379-1389, 1981.
13. Kobrin MS, Asa SL, Samsoondar J, Kudlow J. α-transforming growth factor in the bovine anterior pituitary gland: secretion by dispersed cells and immunohistochemical localization. Endocrinology 121:1412-1416, 1987.
14. Kasselberg AG, Orth DN, Gray ME, Stahlman MT. Immunocytochemical localization of human epidermal growth factor/urogastrone in several human tissues. J Histochem Cytochem 33:315-322, 1985.
15. Kirschner MA. The role of hormones in the etiology of human breast cancer. Cancer 39:2716-2726, 1977.
16. Salmi T. Risk factors in endometrial carcinoma with special reference to the use of estrogens. Acta Obstet Gynecol Scand 86 (suppl): 1-119, 1979.
17. Smith JA Jr. New methods of endocrine manage-

ment of prostatic cancer. J Urol 137:1-10, 1987.
18. Williams ED. The aetiology of thyroid tumours. Clin Endocrinol Metab 8:193-207, 1979.
19. Neville AM, O'Hare MJ. The Human Adrenal Cortex. Pathology and Biology - An Integrated Approach. Berlin: Springer-Verlag, 1982.
20. Furth J, Ueda G, Clifton KH. The pathophysiology of pituitaries and their tumors: methodologic advances. In: Busch H, Ed. Methods in Cancer Research. New York: Academic Press, vol X, pp 201-277, 1973.
21. Newell ME, Lippe BM, Ehrlich RM. Testicular tumors associated with congenital adrenal hyperplasia: A continuing diagnostic and therapeutic dilemma. J Urol 117:256-258, 1977.
22. Neubecker RD, Theiss EA. Sertoli cell adenomas in patients with testicular feminization. AmJ Clin Pathol 38:52-59, 1962.
23. Akerström G, Malmaeus J, Grimelius L, Ljunghall S, Bergström R. Histological changes in parathyroid glands in subclinical and clinical renal disease. An autopsy investigation. Scand J Urol Nephrol 18:75-84, 1984.

Received October 30, 1989
Accepted January 9, 1990