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Nitric Oxide Synthase in the Human Pituitary Gland

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Nitric oxide (NO) is generated by the NO synthase family of isozymes, which is present in many mammalian cells. The constitutive NO synthase isozymes generate NO, which acts via signal transduction mechanisms in the regulation of many functions including vascular tone and blood pressure, and the inducible isozymes mediate immunological mechanisms by cytotoxic and cytostatic effects. To determine whether NO has a role in anterior pituitary cell function, immunohistochemistry and in situ hybridization analyses were used to study NO synthase expression in normal and neoplastic human pituitary tissues. Brain NO synthase was localized in the anterior pituitary in secretory and in folliculo-stellate cells and in the posterior pituitary. Pituitary adenomas had higher levels of brain NO synthase protein and mRNA compared with normal pituitaries. Endothelial NO synthase was also present in anterior and posterior pituitary cells and in endothelial cells of the pituitary. Immunoblotting studies with brain NO synthase antibodies detected a slowly migrating ~155-kd band and more rapidly migrating ~90-kd and ~60-kd bands. Endothelial NO synthase, but not macrophage NO synthase, was also detected in the pituitary by immunoblotting studies, confirming the immunohistochemical observations. These findings indicate that NO synthase is expressed in normal and neoplastic human pituitary tissues with increased levels of brain NO synthase protein and mRNA in adenomas compared with non-neoplastic pituitary cells and suggest that NO may play a regulatory role in hormone secretion in anterior pituitary cells. (Am J Pathol 1995, 146:86–94)

The nitric oxide (NO) synthases are a group of isozymes that convert L-arginine to L-citrulline and generate NO.[1–5] The major forms of NO synthase include 1) a constitutive calcium- and calmodulin-dependent form that does not require tetrahydrobiopterin for activity, and 2) an inducible form that requires tetrahydrobiopterin for activity and is independent of calcium and calmodulin for regulation. The constitutive enzyme is present in endothelium, adrenal gland, brain, and platelets, and the inducible form is present in macrophages, hepatocytes, and vascular smooth muscle and endothelial cells.[1] The constitutive form of NO synthase generates low concentrations of NO, which is important in signal transduction mechanisms including the regulation of basal vascular tone and blood pressure,[5] whereas the inducible form, which can be induced by endotoxin and some cytokines and is inhibited by glucocorticoids, may have important roles in generating large amounts of NO with cytotoxic/cytostatic actions.[6]

NO synthase has been localized in human tissues in the central nervous system, endothelial cells, and macrophages,[7] and a recent study has examined NO synthase in human gynecological malignancies.[8] Various NO synthase genes have been cloned including the constitutive neuronal[9,10] and endothelial forms[11] and the inducible NO synthase present in macrophages.[12]

A few studies have examined NO synthase in rat and mouse pituitary tissues.[13–16] The studies performed on normal rat pituitary suggested that NO synthase may regulate hormone release in specific pituitary cell types.[15,16] The human anterior pituitary and tumors derived from anterior pituitary cells represent a heterogeneous group of cells and tumor types that respond to different stimulatory and inhibitory signals.[17] Analysis of the expression of NO synthase in normal and neoplastic pituitary and the distribution of NO synthase in various pituitary cell types should provide information about the role of NO in human pituitary function. In this report we examine the

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localization of NO synthase in normal and neoplastic pituitary tissues by immunohistochemistry and *in situ* hybridization (ISH).

Materials and Methods

Tissues

Pituitary tumors from 32 patients operated on at the Mayo Clinic between 1986 and 1993 were used for these studies. Formalin-fixed paraffin-embedded blocks from these cases were used for immunohistochemistry and *in situ* hybridization analyses. The tumors were characterized by immunohistochemistry with antibodies against prolactin (PRL), growth hormone (GH), adrenocorticotrophic hormone (ACTH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), and thyroid-stimulating hormone (TSH). In addition, all null cell and gonadotroph adenomas were examined by electron microscopy. Null cell adenomas contained <25% FSH or LH positive cells after immunostaining and were negative for all other pituitary hormones. Four non-neoplastic pituitaries were obtained postmortem within 6 hours of death. The latter patients had no known endocrine diseases.

Protein from five additional pituitary adenomas, one non-neoplastic pituitary and cerebral cortex tissues, were extracted in the presence of proteinase inhibitors in phosphate-buffered saline as previously reported[18] and used for immunoblot analyses.

Using previously described methods[19] a null cell adenoma and a prolactinoma from two additional patients were dissociated and cultured for 7 days. Thereafter cytospin preparations were made for immunohistochemical and *in situ* hybridization studies.

Immunohistochemistry

A polyclonal antiserum to brain NO synthase (B220–1) was obtained from Euro-Diagnostica (Malmö, Sweden). The antiserum was produced in rabbit, the immunogen being a synthetic peptide from the C-terminal of the cloned rat cerebellar NO synthase linked to bovine serum albumin.[20] It was used at a 1:500 dilution. A monoclonal and a polyclonal (used at a 1:50 dilution) antibody against brain NO synthase, a monoclonal antibody against endothelial NO synthase (used at 1:500 dilution), and a monoclonal antibody against macrophage NO synthase (used at a 1:20 dilution) were obtained from Transduction Laboratories (Lexington, KY). All of these antibodies were produced using synthetic peptides. The monoclonal brain NO synthase was not effective in staining paraffin tissue sections, but worked well in immunoblotting studies (see below). Antibodies for S-100 acidic protein was from Dako Corp. (Carpinteria, CA; used at a 1:2000 dilution). Antibodies directed toward pituitary hormones were donated by the National Hormone and Pituitary Program (Rockville, MD) and were used as previously described.[18]

Using the avidin biotin complex method with kits from Vector Labs (Burlingame, CA), staining for NO synthase and S-100 protein was performed as previously reported.[19] Localization of NO synthase and S-100 protein in the same tissue section was performed sequentially using peroxidase with diaminobenzidine chromogen and alkaline phosphate enzyme with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) kits from Vector Labs to detect the labeled antigens.

Negative controls for immunostaining consisted of substituting normal rabbit serum in place of the primary antiserum. Controls for double staining consisted of localizing S-100 protein and NO synthase with both the peroxidase and with the alkaline phosphatase detection systems on different slides. Omission of primary antisera in the peroxidase and alkaline phosphatase systems served as additional controls.

Immunoblot Analysis

One-dimensional SDS polyacrylamide gel electrophoresis was performed using 7% gel with the discontinuous buffer system of Laemmli as previously reported.[19,21] The electrophoresed proteins were transferred onto PVDF membrane (BioRad, Hercules, CA) and subjected to immunoblot analysis using antisera to brain NO synthase (monoclonal antibody used at 1:250 dilution and the polyclonal antiserum at 1:250 dilution); endothelial NO synthase (1:2000 dilution) and macrophage NO synthase (1:250 dilution) and detected with the alkaline phosphatase NBT BCIP system.

Samples of brain (extracted in our laboratory), endothelial cell, and macrophages (purchased from Transduction Laboratories) were used as positive controls for immunoblot analyses. Molecular weight standards were obtained from BioRad.

In Situ *Hybridization*

The probes for ISH were synthesized from the published sequence of brain NO synthase[9] using a Model 394 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA) in the Biochemistry Department at Mayo Foundation. The following probes were used: antisense one, 5′ CAA CTT GGC GTC ATC TGC TCA TTC

CGA TTC (nucleotides 211–240); antisense two: 5′ TGC TGA GGG CCA TTA CCC AGA CCT GTC ACT (nucleotides 801–830); sense probe: 5′ CGG CTC AGC AAG CCT CCC GTG ATC ATC TCA (nucleotides 151–160). The specificity of the probes was confirmed by checking GeneBank (Madison, Wisconsin) and by the experiments described below.

In situ hybridization and cytospin preparations on paraffin sections was performed as previously reported.[18,22] Probes were labeled with ^{35}S-dATP; antisense probes were used as a cocktail with 3×10^6 cpm/slide of each probe. The sense probe was used as a negative control. Other controls consisted of pretreating tissues with ribonuclease A (250 μg/ml) for 2 hours at 37 C before hybridizing with the probe. Before hybridization the specificity of the probes was confirmed by competition studies with a 100-fold excess of unlabeled probe. Additional *in situ* hybridization analyses for specificity was done with frozen sections of brain tissues.

Immunostaining was semiquantitatively graded as 1+ for weak, 2+ for moderate, and 3+ for strong staining. The degree of positivity of the ISH reaction was evaluated by counting the number of grains per cell, 1+ representing <10; 2+, 10 to 15; and 3+, >15 above background level.

Results

Immunohistochemistry

Immunohistochemistry showed staining of some cells in the normal anterior pituitary with the rabbit polyclonal anti-brain NO synthase antibody. Many folliculo-stellate cells, identified by their irregular cytoplasmic processes and by staining for S-100 protein, were also positive for NO synthase. Staining was present both in the normal pituitary and within residual folliculo-stellate cells in pituitary tumors (Figure 1). Colocalization of brain NO synthase and S-100 protein in the same cells, as demonstrated by double staining, confirmed that it was folliculo-stellate cells expressed NO synthase (Figure 1). Immunostaining of adenomas accompanied by non-neoplastic pituitary tissue as in six surgically resected tumors and in an autopsy pituitary revealed more intense staining in the adenomas than in non-neoplastic anterior pituitary tissues (Figure 2). The posterior pituitary tissues were also positive for brain NO synthase, especially within dilated axon terminals (Herring bodies) (data not shown). Endothelial cells in the anterior pituitary were weakly immunopositive for brain NO synthase.

Immunostaining with another polyclonal antiserum for brain NO synthase produced similar results in non-neoplastic pituitary. Weak positive immunoreactivity was present with the monoclonal anti-endothelial NO synthase antibody in the anterior pituitary with stronger staining of endothelial cells. Anti-macrophage NO synthase was negative in the pituitary.

Immunohistochemical staining of pituitary adenomas showed positive staining in all tumor groups with two different anti-brain NO synthase polyclonal antisera. Reactivity varied from weak staining in glycoprotein-producing and null cell adenomas to stronger staining in PRL, GH, and ACTH adenomas (Figure 3) (Table 1). Of all tumor groups, prolactinomas had the strongest immunoreactivity with the anti-brain NO synthase antisera (Table 1). The monoclonal anti-brain antibody did not stain the paraffin tissue sections. Anti-endothelial NO synthase was weakly positive in most groups of adenomas (Table 1) with stronger staining of endothelial cells (Figure 4). The adenomas were negative with the anti-macrophage NO synthase antibody. Negative controls with omission of the primary antisera were consistently negative.

Immunoblot Analysis

Immunoblotting with all three brain NO synthase antibodies resulted in several bands including a slowly migrating ~155-kd band, and more rapidly migrating ~90- and ~60-kd bands (Figure 5A). The 60-kd band stained most intensely with all three antibodies. Although the intensity of the bands in the neoplastic and non-neoplastic pituitaries were similar with the polyclonal antiserum, the monoclonal anti-brain NO synthase resulted in more intense ~155- and ~60-kd bands in adenomas compared with the non-neoplastic pituitary (Figure 5B). The endothelial NO synthase antibody produced weakly stained bands of ~140, ~70, and ~50 kd in all pituitary tissues, whereas the positive endothelial cell controls had an intensely staining ~140-kd band and less intensely staining ~70-kd bands (data not shown). Immunoblotting with the anti-macrophage NO synthase antibody was negative in the pituitary tissues, and the positive control cells had a strongly staining ~130-kd band (data not shown).

In Situ *Hybridization*

The specificity of the ISH reaction with the oligonucleotide probes was analyzed by various methods. 1) The use of the sense probe resulted in a background hybridization signal (Figure 4). 2) Ribonuclease A

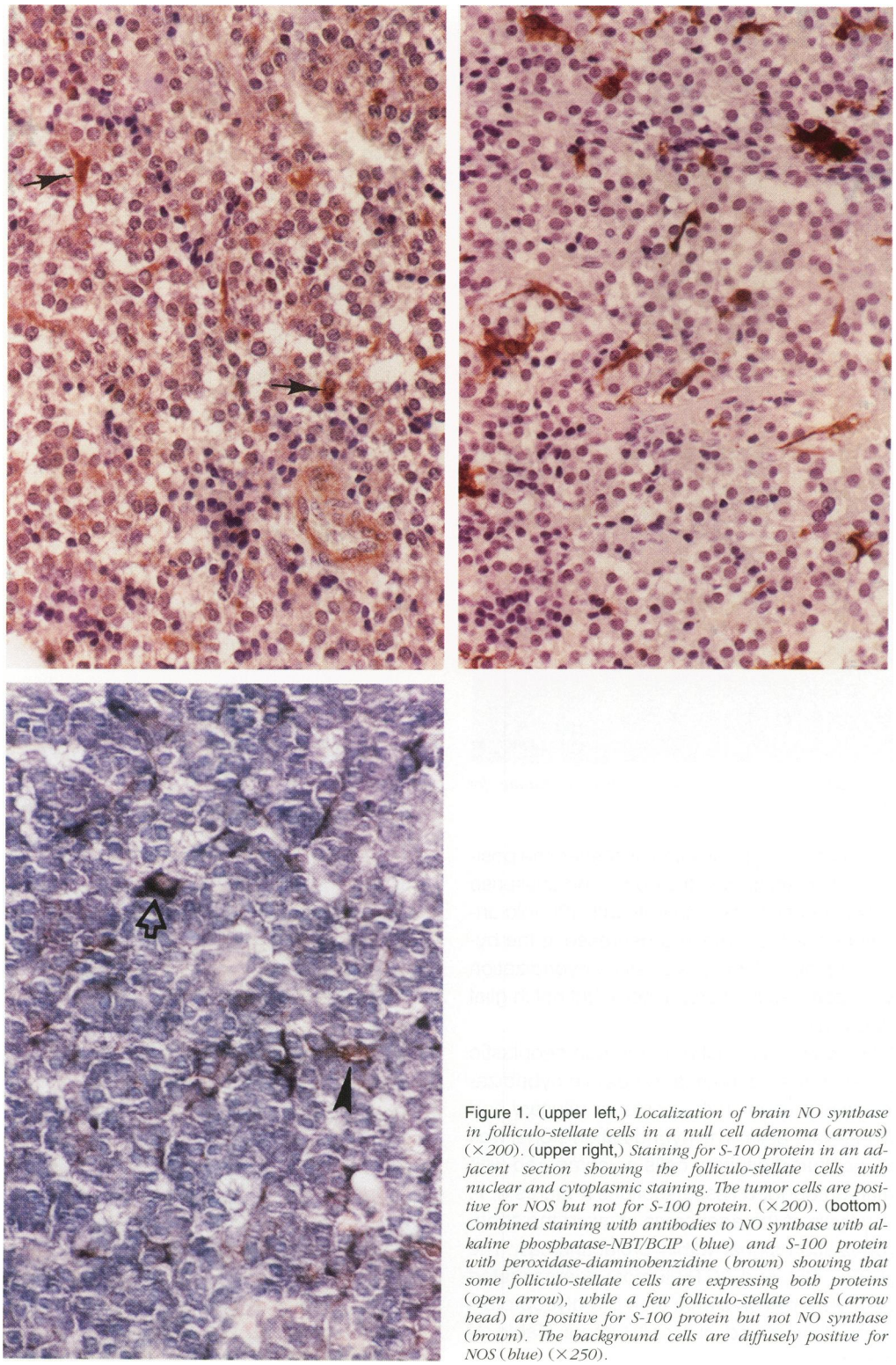

Figure 1. (upper left,) *Localization of brain NO synthase in folliculo-stellate cells in a null cell adenoma (arrows) (×200).* (upper right,) *Staining for S-100 protein in an adjacent section showing the folliculo-stellate cells with nuclear and cytoplasmic staining. The tumor cells are positive for NOS but not for S-100 protein. (×200).* (bottom) *Combined staining with antibodies to NO synthase with alkaline phosphatase-NBT/BCIP (blue) and S-100 protein with peroxidase-diaminobenzidine (brown) showing that some folliculo-stellate cells are expressing both proteins (open arrow), while a few folliculo-stellate cells (arrow head) are positive for S-100 protein but not NO synthase (brown). The background cells are diffusely positive for NOS (blue) (×250).*

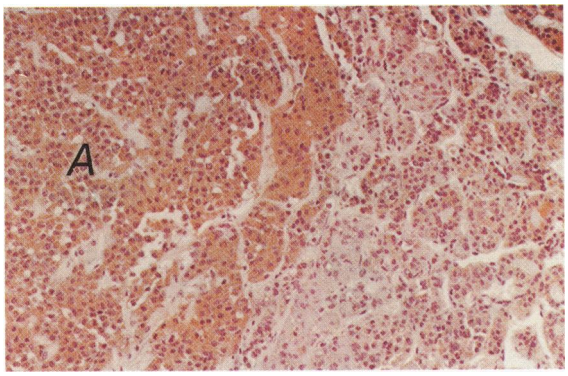

Figure 2. *Null cell adenoma* (A) *and adjacent non-neoplastic pituitary showing stronger staining for brain NO synthase in the tumor cells* (×200).

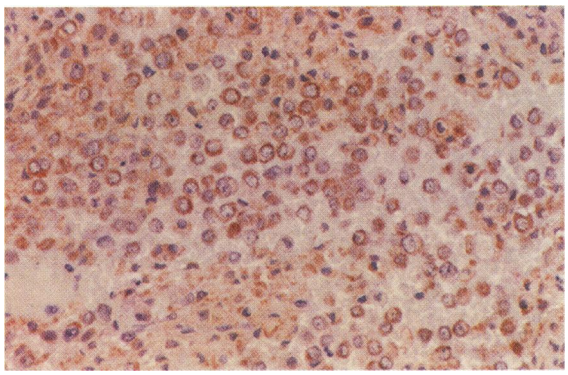

Figure 3. *ACTH adenoma showing strong cytoplasmic staining for brain NO synthase* (×250).

pretreatment before hybridization abolished the positive hybridization signal with the combined antisense probes. 3) Competition experiments with 100-fold unlabeled probes led to a marked decrease in the hybridization signal. 4) Finally, a positive hybridization signal was observed in neuronal cells, but not in glial cells of the brain.

The ISH signal was weaker in the non-neoplastic pituitary than in the adenomas. A positive hybridization signal was present in the posterior pituitary and in folliculo-stellate cells. The hybridization signal was stronger in cultured pituitary cells as compared with the paraffin-embedded tissue sections (Figures 6, 7, and 8). Analysis of the adenomas showed the strongest hybridization signal within prolactinomas. The group of PRL, GH, and ACTH tumors demonstrated a stronger hybridization signal than did glycoprotein hormone-producing and null cell tumors (Table 1) (Figures 6–8).

Discussion

These immunohistochemical immunoblotting and ISH analyses provide evidence for the presence of brain

Table 1. *Localization of Nitric Oxide Synthase (NOS) in Pituitary Adenomas by Immunohistochemistry (IHC) and In Situ Hybridization (ISH)*

Case	Tumor type	IHC* Brain NOS	IHC* Endo NOS	ISH Brain NOS
1	Prolactinoma	2+	1+	2+
2	Prolactinoma	3+	1+	2+
3	Prolactinoma	2+	3+	2+
4	Prolactinoma	2+	1+	3+
5	Prolactinoma	1+	1+	3+
6	Prolactinoma	3+	1+	3+
7	Prolactinoma	2+	0	2+
8	GH adenoma	1+	3+	2+
9	GH adenoma	2+	2+	2+
10	GH adenoma	2+	1+	2+
11	ACTH adenoma	2+	1+	3+
12	ACTH adenoma	2+	0	1+
13	ACTH adenoma	2+	1+	1+
14	ACTH adenoma	3+	2+	2+
15	ACTH adenoma	2+	2+	2+
16	TSH adenoma	1+	1+	1+
17	TSH adenoma	1+	0	1+
18	TSH adenoma	1+	1+	2+
19	GTH adenoma	1+	1+	2+
20	GTH adenoma	2+	1+	2+
21	GTH adenoma	1+	2+	2+
22	GTH adenoma	2+	2+	1+
23	GTH adenoma	1+	1+	2+
24	GTH adenoma	1+	1+	1+
25	Null Cell adenoma	2+	1+	2+
26	Null Cell adenoma	1+	3+	2+
27	Null Cell adenoma	1+	0	1+
28	Null Cell adenoma	1+	1+	2+
29	Null Cell adenoma	1+	2+	1+
30	Plurihormonal adenoma	2+	0	2+
31	Plurihormonal adenoma	1+	1+	1+
32	Plurihormonal adenoma	1+	0	1+

IHC: 0 = absent staining; 1+ = weak; 2+ = moderate; 3+ = strong immunoreactivity

ISH: 1+ < 10; 2+ 10–15; 3+ > 15 grains per cell.

*The anti-brain NO synthase results are with the Euro-Diagnostica antibody and the anti-endothelial NO synthase results are with the Transduction Laboratory antibody.

and endothelial NO synthase in normal and neoplastic human pituitary tissues. Anterior and posterior pituitary cells, folliculo-stellate cells, and pituitary tumors all expressed NO synthase. The detection of brain NO synthase mRNA in these tissues by ISH indicates that this enzyme is produced by these cells. The recent studies of NO synthase in rat pituitary tissues by Caccatelli et al[15] differed somewhat from our findings, as they found NO synthase primarily in the folliculo-stellate cells and gonadotroph cells. Although prolactinomas appear to have the highest levels of NO synthase protein and mRNA in our study, NO synthase appears to be present in all normal and neoplastic pituitary cell types.

When non-neoplastic and tumor tissues were examined on the same slide, the tumors had higher levels of NO synthase protein and mRNA expression,

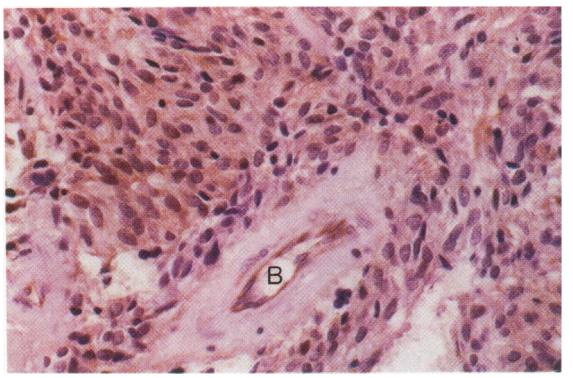

Figure 4. *PRL adenoma showing strong (3+) cytoplasmic staining of endothelial cells for endothelial NO synthase (arrows) and weak (1+) cytoplasmic immunoreactivity in the tumor cells (×300).*

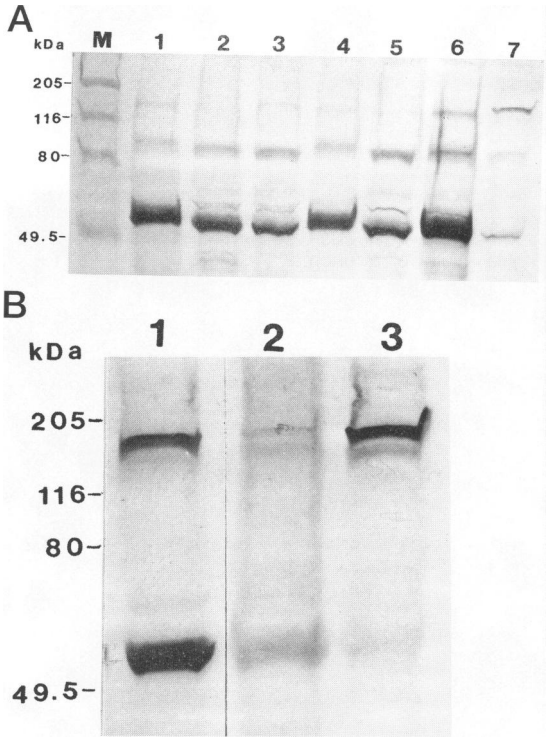

Figure 5. *Immunoblot analysis of pituitary tissues. Tissues were subjected to one-dimensional SDS-electrophoresis followed by immunoblotting. Fifty μg of protein per lane was used.* (A) *Immunoblotting with a polyclonal antiserum to brain NO synthase M, molecular weight markers. Lane 1, GH adenoma; lane 2, PRL adenoma; lane 3, ACTH adenoma; lane 4, gonadotroph adenoma; lane 5, null cell adenoma; lane 6, non-neoplastic pituitary; lane 7, human cerebral cortex standard.* (B) *Immunoblotting with a monoclonal antibody to brain NO synthase. Lane 1, ACTH adenoma; lane 2, non-neoplastic pituitary; lane 3, human cerebral cortex standard.*

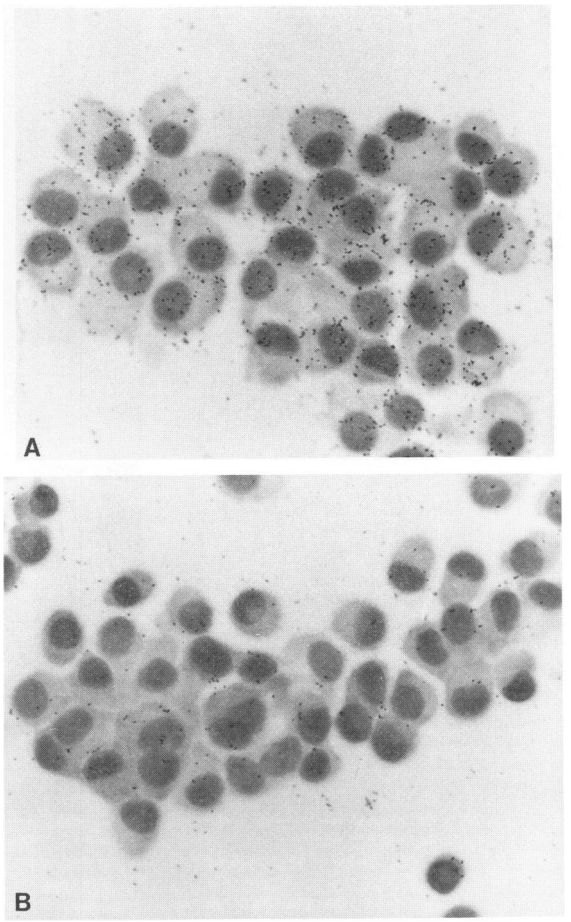

Figure 6. (A) In situ *hybridization to localize brain NO synthase mRNA in cultured cells of a prolactinoma. (×400)* (B) *Hybridization with the sense probe shows only a background hybridization signal (×400).*

indicating that this enzyme is expressed in higher levels in pituitary tumors. These differences were also observed in immunoblotting study with the monoclonal brain NO synthase antibody. In a recent study of normal and malignant gynecological tissues Thompsen et al[8] found NO synthase expression only in malignant tissues, thus suggesting that NO synthase activity was related to malignancy. Furthermore, they suggested that the activity may also be related to tumor grade, given that they observed that poorly differentiated ovarian carcinomas had higher levels of NO synthase activity than moderately differentiated ones. Our findings in the pituitary show that brain NO synthase is present in non-neoplastic as well as neoplastic pituitary cells but that the levels are higher in adenomas. In keeping with the suggestion of Thompson et al[8] these results also suggest increased expression during neoplastic development.

The presence of low molecular weight bands of ~50 to ~90 kd, in addition to the ~155- and ~140-kd bands for brain and endothelial NO synthase, respectively, suggests that the low molecular weight bands may be degradation products of the ~155- and ~140-kd bands, which is in agreement with the findings of Springall et al.[7]

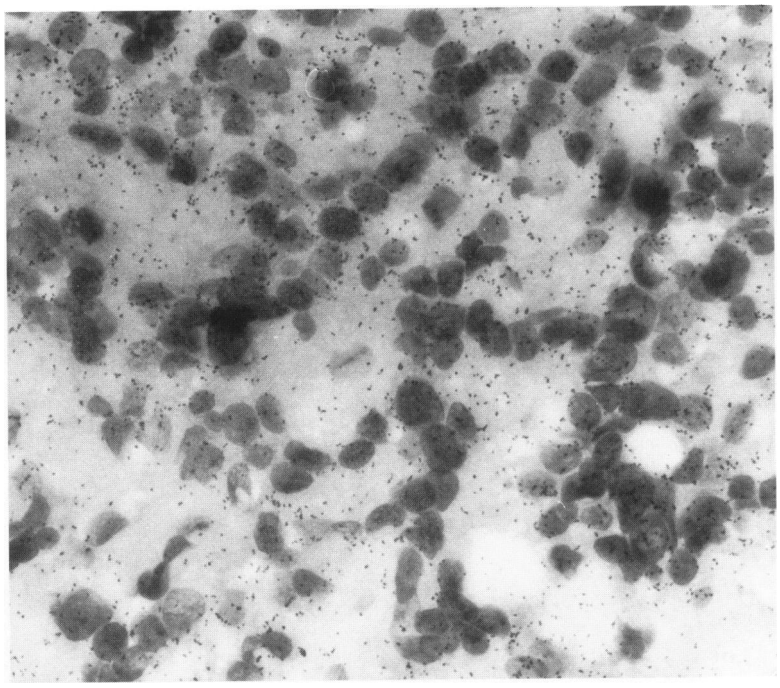

Figure 7. *Paraffin section of a prolactin-producing pituitary tumor hybridized with brain NO synthase probe cocktail showing a strong (3+) positive signal over the tumor cells (×350).*

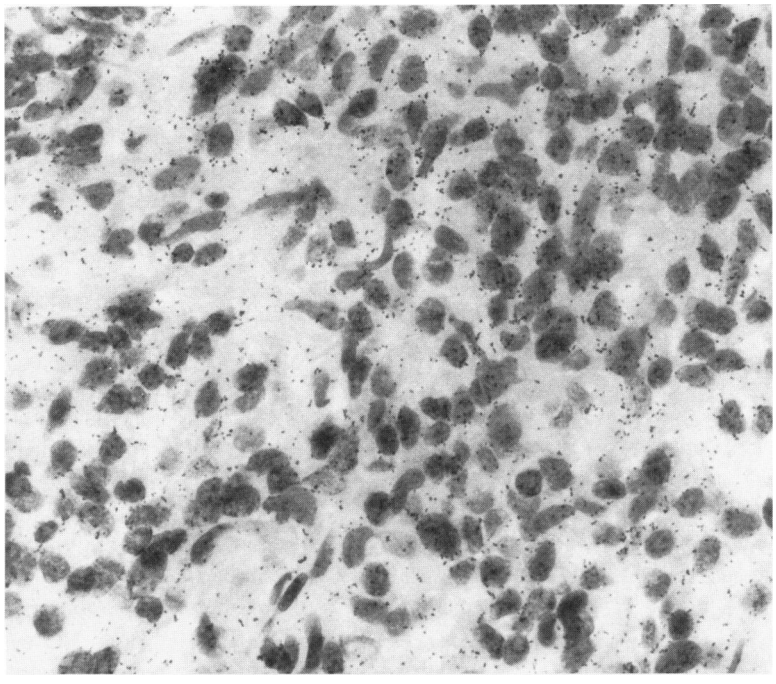

Figure 8. *Paraffin section of an ACTH-producing tumor hybridized with brain NO synthase probe cocktail showing a moderate (2+) positive signal over the tumor cells (×300).*

Our observations that prolactinomas had slightly higher levels of brain NO synthase suggest that this enzyme may play a significant role in regulating this subtype of pituitary adenoma. Kato[15] observed that NO in the rat pituitary gland inhibited growth hormone releasing hormone-induced, but not basal, release of GH, suggesting a role of NO in regulating GH secretion. Prolactinoma and normal prolactin cells are a unique cell type, given that dopamine, the principal regulatory stimulus from the hypothalamus, exerts an inhibitory effect upon prolactin secretion.[17] Thus, NO may have a direct effect upon PRL secretion or an indirect effect via the folliculo-stellate cell. Folliculo-stellate cells, which do not contain secretory granules, appear to play numerous roles in the regulation of anterior pituitary cell function.[23–28] Various substances including basic fibroblast growth factor, cytokines, vascular endothelial growth factor, S-100 pro-

tein, and follistatin (an FSH release-inhibitory peptide) have been identified in folliculo-stellate cells of various species.[23–28] Our observations in the human and the recent studies in the rat pituitary of Caccatelli et al[15] indicate that brain NO synthase in folliculo-stellate cell may exert a regulatory function, one of controlling secretion in other anterior pituitary cell types.

Our immunohistochemical studies indicated that the NO synthase antiserum prepared from rat cerebellum also reacted with endothelial cells. We also observed endothelial NO synthase expression in non-neoplastic and tumorous anterior pituitary cells. Although brain NO synthase shows 52% amino acid identity to endothelial NO synthase, which may lead to antibody cross reactivity, the immunoblot studies suggested that these antibodies were recognizing different NO synthase isozymes. Recent studies by Dinerman et al[29] utilizing highly specific antibodies to endothelial NO synthase showed this isozyme to be present in neuronal populations in the brain, therefore indicating a broader distribution of these constitutive forms of NO synthase than was previously realized.[29] Ohta et al[14] examined NO production in the mouse AtT 20/D16 pituitary tumor cell line and showed that both cytokine-inducible and calcium-calmodulin-independent NOS were present in these tumors indicating a more widespread distribution of the isozymes.

In summary, these studies indicate that anterior pituitary secretory cells, folliculo-stellate cells, and the terminations of hypothalamic neurons within the posterior pituitary express brain and endothelial NO synthase. Brain NO synthase is present in normal and neoplastic human pituitaries. The observation that higher levels are expressed in adenomas suggests increased expression during neoplastic development. These results suggest that NO synthase may play a role in regulating pituitary cell function.

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