

Adrenomedullin expression in pituitary adenomas and nontumoral adenohypophyses

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Summary. Adrenomedullin (ADM) is a novel peptide originally identified in extracts of human pheochromocytoma. It is produced by several tissues, including the pituitary gland. The presence of ADM has been immunohistochemically demonstrated in pathologic pituitary glands, but no systematic study of ADM expression in human pituitary adenomas has been reported. Thus, we investigated ADM immunoreactivity in 88 various hormone-secreting and clinically nonfunctioning pituitary adenoma types as well as 30 nontumoral adenohypophyses. Furthermore, ADM immunoreactivity was assessed on a 0 to +3 scale in all samples. We found strong immunoreactivity for ADM in normal gonadotrophs also expressing FSH and LH whereas in the other adenohypophysial cell types expression of ADM was mild. Results showed that normal adenohypophyses were strongly immunopositive for ADM (2.18±0.11). Our findings demonstrate that ADM expression in the anterior pituitary is diminished in tumors as compared to the normal gland. The physiologic function of ADM is unknown, but it could act as a paracrine or autocrine factor in the adenohypophysis.

Key words: Adenohypophysis, Adrenomedullin, Immunohistochemistry, Pathology, Pituitary adenoma

Introduction

Adrenomedullin (ADM), a potent vasodilator peptide, originally isolated from human adrenal pheochromocytoma cells by Kitamura et al. (1993), has multiple regulatory functions, vasodilation and hypotensive effects being principle among them (Imai et

al., 1995; Hinson et al., 2000). Although its effects include an even wider range of actions, e.g. regulating cellular growth and differentiation, modulating hormone secretion, antimicrobial activity, etc (Imai et al., 1995), ADM is produced by most cells and is derived from proADM, a large precursor molecule converted after cleavage to proADM, a precursor of mature ADM. Human ADM consists of 52 amino acids and its structure is homologous to calcitonin gene-related peptide (CGRP), calcitonin and amylin, all members of the same peptide family (Muff et al., 1995; Beltowski and Jamroz, 2004).

According to Beltowski and Jamroz (2004), ADM is not stored in secretory granules (except in pancreatic endocrine cells) and is released immediately after synthesis. However, proadrenomedullin was detected in the secretory granules of gonadotrophs of the mammalian pituitary and is colocalized with FSH (Montuenga et al., 2000). ADM exerts its effects via specific cell surface receptors (usually ADM-R) but also via receptors for CGRP, (Hofbauer et al., 2000) a structurally related peptide stimulating two signal transduction pathways, cAMP accumulation and Ca²⁺ mobilization (Shimekake et al., 1995). ADM circulates in plasma in picomolar concentrations (Letizia et al., 2000, 2003a,b) and is increased in various diseases e.g. cardiovascular, respiratory, hepatic and renal disorders (Cheung and Leung, 1997).

The biological activity of ADM in the cardiovascular system is well documented in the reviews of Hinson et al. (2000) and of Beltowski and Jamroz (2004). It seems that an increase in circulating ADM in various diseases is a consequence rather than a cause of the pathology. Furthermore, it is unclear whether systemic increases in ADM result from local sites of production and action or whether increased plasma ADM functions as a hormone to cause a general decrease in vascular resistance and a reduction in blood pressure (Hinson et al., 2000).

It was reported that the principal site of ADM synthesis is the endothelial cell. These cells have the highest expression levels of ADM in the entire body and account for the majority of ADM released to the blood. Clinical studies of the production and clearance of circulating ADM in humans (Nishikimi et al., 1994) found it not only in endothelial cells but in a variety of cells in the cardiovascular, renal, respiratory, reproductive and gastrointestinal systems. Skin and fibroblasts (Hinson et al., 2000) as well as brain, particularly the thalamus and hypothalamus (Serrano et al., 2002), also contain ADM. It has also been identified in endocrine organs, including the parathyroid glands (Letizia et al., 2004), pancreatic islets (Washimine et al., 1995; Letizia et al., 2001), adrenal cortex and medulla (Sato et al., 1995; Kapas and Hinson, 2002), the gastrointestinal neuroendocrine system (Washimine et al., 1995), placenta (Letizia et al., 2003b), and the pituitary gland (Washimine et al., 1995; Takahashi et al., 1997; Montuenga et al., 2000; Serrano et al., 2002; Letizia et al., 2003b). High concentration of immunoreactive ADM were present in whole pituitaries (Takahashi et al., 1997), suggesting that it could act as a neuromodulator or neurotransmitter in the brain and as an autocrine paracrine factor or hormone in the pituitary.

Most of the studies of ADM in the pituitary relate to its role in regulating the hypothalamus-pituitary-adrenocortical (HPA) axis. Thus, corticotrophs are the cells most investigated. In 1995, Samson et al. found ADM to inhibit adrenocorticotropin (ACTH) secretion from dispersed rat anterior pituitary cells. Since then, many investigations have been published describing the regulatory actions of ADM upon the HPA (Nussdorfer et al., 1997; Knerr et al., 2001; Shan and Krukoff, 2001; Taylor and Samson, 2004). The secretion of GH and PRL in the adenohipophysis was also studied since their release is known to be altered by stress (Taylor and Samson, 2004). It was concluded that in rats ADM acts

upon the brain to stimulate the secretion of both PRL and ACTH and to inhibit the secretion of GH (Taylor and Samson, 2004).

With regard to tumors, ADM expression has also been investigated in the human endocrine system. Tumors studied include insulinomas (Letizia et al., 2001), parathyroid proliferations (Letizia et al., 2004), adrenocortical tumors (Takahashi et al., 1998) and pituitary adenomas, both functioning and non-hormone-secreting (Knerr et al., 2001).

Materials and methods

Seventy-four endocrinologically active pituitary adenomas and fourteen clinically non-functioning pituitary adenomas were retrieved from the files of St. Michael's Hospital. Among endocrinologically active adenomas, thirty-seven corticotroph cell adenomas were resected from patients with Cushing's disease. In addition 30 autopsy-obtained, nontumoral pituitaries were also investigated (Table 1). Several slides containing the tumor included also adjacent nontumoral areas. These areas were also assessed.

All adenomas including corticotroph cell adenomas were removed at transsphenoidal surgery. Clinical data was available on 27 of the 37 patients, including five men (mean age 30.5 years, range 15-48, SEM 5.9) and 22 women (mean age 38 years, range 19-58, SEM 2.3). The REB of St. Michael's Hospital approved our project and the use of human specimens. Tumors were nonrecurring in males, while those of occurring in females, one recurred, one was periodically active, and one was persistent.

All specimens were fixed in 10% buffered formalin, routinely processed, paraffin embedded, cut at 4-6 μ m, and stained with hematoxylin and eosin (H&E), periodic acid-Schiff and the Gordon-Sweet Silver method to demonstrate reticulin fibers. Immunostaining employed

Table 1. Expression of adrenomedullin in pituitary adenomas and nontumoral adenohipophysis.

Adenoma Subtypes	Number of samples (N)	Mean ADM Staining †	SEM
Tumoral adenohipophysis			
GH cell	4	0.56 ^{at}	0.19
PRL cell	5	0.80 ^a	0.24
GH cell and PRL cell	4	0.50 ^a	0.29
Mammotroph cell	4	0.42 ^a	0.25
Corticotroph cell (in Cushing's disease)	37	1.69 ^{ab}	0.13
Silent corticotroph subtype 1	5	0.93 ^{ab}	0.36
Silent corticotroph subtype 2	5	1.63 ^{ab}	0.34
Silent subtype 3	4	0.92 ^{ab}	0.42
Gonadotroph cell	5	1.33 ^{ab}	0.28
TSH cell	4	1.50 ^{ab}	0.40
Null cell, non-oncocyctic	5	0.60 ^a	0.27
Null cell, oncocyctic	6	1.14 ^{ab}	0.32
Nontumoral adenohipophysis	30	2.18 ^b	0.11

†: Means with different letters are significantly different ($P < 0.05$) to Student-Newman-Keuls multiple range test.

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streptavidin-biotin-peroxidase complex method and antisera directed against growth hormone (GH), prolactin (PRL), adrenocorticotrophic hormone (ACTH), thyroid stimulating hormone (TSH), luteinizing hormone (LH), follicle stimulating hormone (FSH), and alpha subunit. In many surgical cases, electron microscopy had been performed for diagnosis on primarily fixed, routinely processed tissues. Source and dilutions of the antibodies, as well as control procedures were described elsewhere (Kovacs et al., 1995, 1997). For the demonstration of ADM, the same method was applied using an affinity purified goat polyclonal antibody raised in a peptide mapping within an internal region of human origin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:50 in antibody diluent reagent solution (Zymed, San Francisco, CA, USA). Microsections of a human pheochromocytoma served as a positive control, one section being included in each staining run. Replacement of the primary antibody with PBS served as a negative control.

Double immunostaining for the full spectrum of adenohipophysial hormones and ADM was carried out to determine which cell type(s) were ADM-producing. The method used DAB as the first chromogen and a VIP SK-4600 kit (Vector Laboratories Inc, Burlingame, CA, USA) for the second. Nuclei were then counterstained with Methyl Green for 20 minutes.

Four unbiased observers assessed the slides in a semiquantitative way, scoring ADM immunopositivity as follows: 0 (completely negative), +1 (slightly positive), +2 (moderately positive) and +3 (strongly positive). Reactions were considered positive when cytoplasmic staining for ADM was observed without background staining. Scores were expressed in terms of mean \pm SEM (standard error of the mean). An analysis of variance (ANOVA) was conducted to estimate the differences between the tumoral and nontumoral groups, followed by a Student-Newman-Keuls multiple range test.

Results

Scores for ADM immunostaining are summarized in Table 1 and Fig. 1. A more extensive information about the expression of ADM in corticotroph adenomas is shown in table 2.

We found that nontumoral pituitaries showed a

strong immunopositivity for ADM achieving the highest scores (2.18 ± 0.11) (Fig. 2) and displaying a mild diffuse pattern with many stronger staining cells randomly distributed. There was no granular staining in the cytoplasm of the cells. On double immunostaining we observed that ADM co-localized mainly with FSH and LH showing a strong immunopositivity for ADM (Fig. 3) whereas the other cell types exhibited a very mild immunoreactivity for ADM. The results indicate that mainly the gonadotrophs synthesize and store ADM in the human adenohipophysis.

In all the pituitary adenomas studied, ADM immunopositivity was lower than in nontumoral pituitary samples. Reactivity was less intense and showed a more diffuse pattern of staining (Fig. 4). There were very significant differences ($P < 0.001$ and $F\text{-value} = 30.392$) between the tumoral (1.27 ± 0.09) and nontumoral groups (2.18 ± 0.11). In comparison, immunopositivity was somewhat decreased in functional and silent subtype 2 corticotroph adenomas (1.69 ± 0.13 and 1.63 ± 0.34), gonadotroph cell adenomas (1.33 ± 0.28) and TSH adenomas (1.50 ± 0.40). In contrast, GH cell adenomas (0.56 ± 0.19), GH and PRL cell adenomas

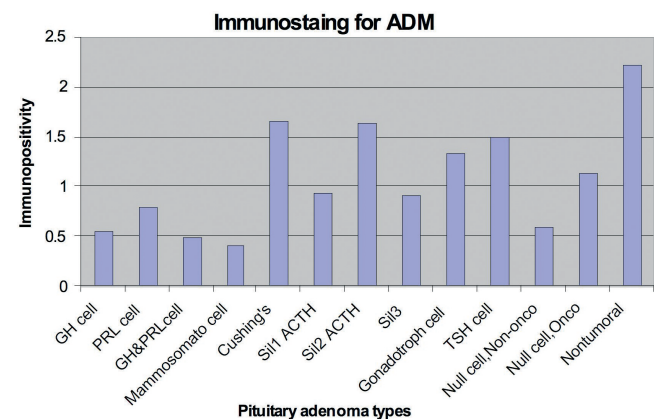


Fig. 1. Graphic displaying the mean \pm SEM of ADM immunoreactivity observed in the range of adenoma types as well as in nontumoral adenohipophysies.

Table 2. Expression of ADM in corticotroph adenomas compared with nontumoral adenohipophysis.

	N	Immunoreactivity				Cumulative Scores		
		Negative	Mildly Positive	Moderately Positive	Strongly Positive	Weak positivity	Moderate/strong positivity	
Scores		0	<1	<1.5	<2	<3	<1.5	>1.5-3
Cushing disease-associated corticotroph adenomas	37	3	6	6	10	12	15	22
Nontumoral adenohipophysis	30	0	3	2	8	17	5	25

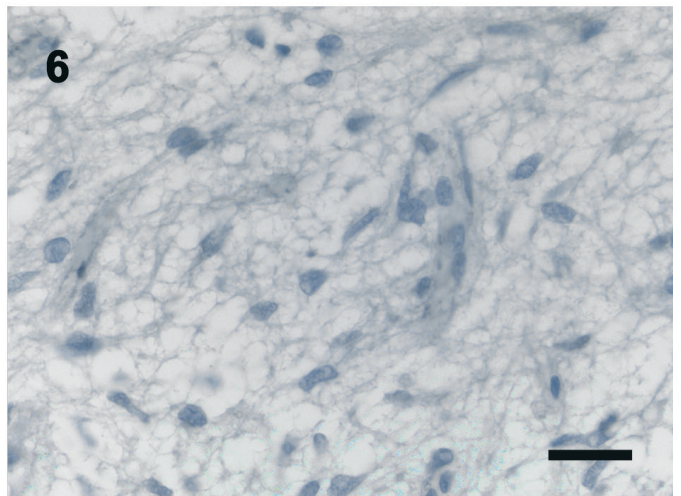
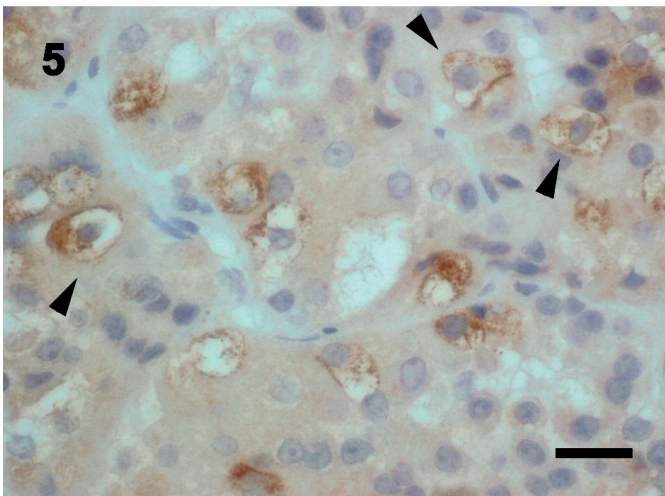
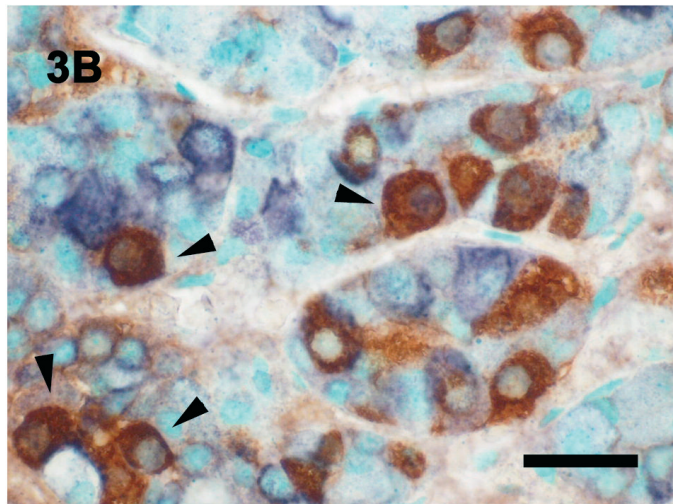
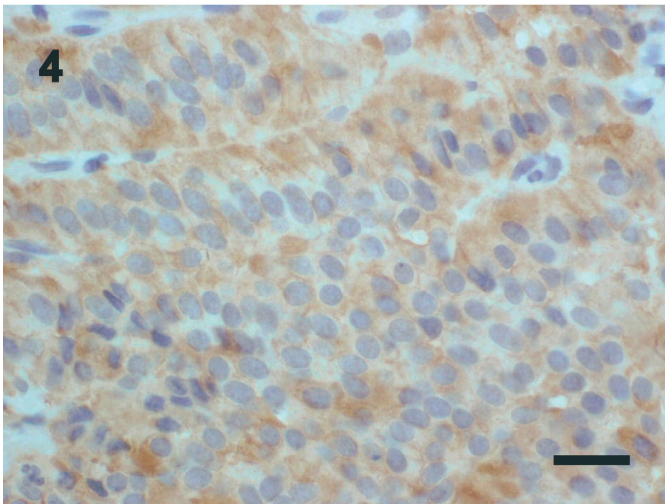
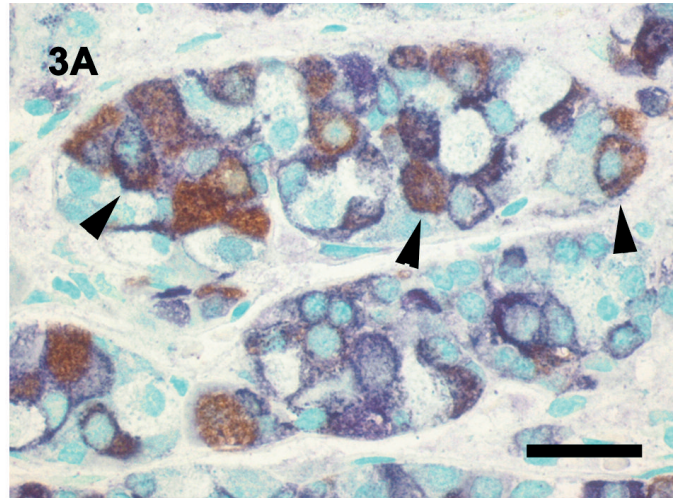
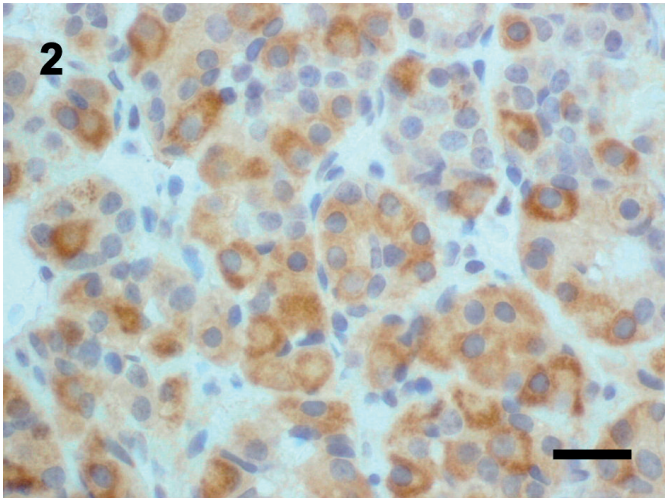


Fig. 2. Nontumoral adenohypophysis showing strong immunoreactivity for ADM. Scale bar: 10 μ m.

Fig. 3. Immunopositivity for ADM shows co-localizing with gonadotroph cells. Double-label immunostaining in nontumoral pituitary. Arrowheads indicate cells double labeled for ADM (blue) and the respective pituitary hormone (brown), FSH (3A) and LH cells (3B). Scale bar: 10 μ m.

Fig. 4. Corticotroph adenoma immunoreactive for ADM. Immunopositivity for ADM is less intense than in nontumoral adenohypophysis (Fig. 2). Scale bar: 10 μ m.

Fig. 5. Photomicrograph of adenohypophysis immunostained for ADM. Many Crooke cells are dispersed throughout the tissue (arrowheads). Scale bar: 10 μ m.

Fig. 6. ADM immunostaining is negative in the neurohypophysis. Scale bar: 10 μ m.

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(0.50 ± 0.29) and mammosomatotroph adenomas (0.42 ± 0.25) showed the lowest levels of ADM immunoreactivity. The statistical analyses for each tumoral type and the nontumoral group confirm that the values of the media were divided into 2 statistically different main subgroups ($p < 0.05$): the lower values (a), and the higher values (b), as well as a third cluster of mean values that shares characteristics with both main subgroups (ab) (Table 1). It is of note that immunopositivity for ADM in gonadotroph adenomas was not as intense as would be expected on the basis of co-localization experiments wherein nontumoral gonadotroph stained strongly.

Considering the Cushing disease-associated corticotroph adenomas in which detailed clinical information was available ($n=27$), ADM immunoreactivity was relatively high in males (2.06 ± 0.36) ($n=5$), approaching the mean value of nontumoral pituitary tissue, and lower in females (1.74 ± 0.18). The degree of ADM expression in the male patients with Cushings' disease did not differ significantly from that of female patients ($P > 0.05$).

Among silent adenomas, clear differences in ADM immunoreactivity were noted among the three types. Staining was much less intense in silent corticotroph adenoma subtype 1 and silent adenoma subtype 3, as compared to silent subtype 2 corticotroph adenoma. Scores in the latter were comparable to mean observed in Cushing disease-associated corticotroph adenomas.

Crooke's cells were present in 7 adenomas of 37 samples of Cushing disease. Three were completely immunonegative for ADM, and four were scored between 1.25 to 3, the sample with most numerous Crooke cells being rated at 2.125 (Fig. 5).

We found that vessels were completely immunonegative for ADM as well as the neurohypophysis included in several sections of nontumoral pituitary (Fig. 6).

Discussion

Our results show that immunopositivity for ADM in nontumoral adeno-hypophysis is quite strong, whereas it is slightly reduced in adenoma samples including those removed from patients with Cushing-associated corticotroph adenomas. On the other hand, as described by Letizia et al. (Letizia et al, 2000), blood ADM concentrations in control patients was 13.7 ± 6.1 pg/mL as compared to 37.6 ± 17.8 pg/m in the setting of Cushing disease due to adenoma (Letizia et al., 2000). Based on these observations, it appears that high adeno-hypophysial ADM immunoreactivity occurs in conjunction with relatively low ADM concentrations in circulating blood. In contrast, patients with Cushing disease whose blood levels of ADM are quite elevated exhibit decreased pituitary immunoreactivity when compared with nontumoral pituitaries.

It has been shown by Samson et al. (1995) that ADM a) inhibits ACTH release from cultured rat anterior pituitary cells in a dose dependent manner and

b) also attenuates CRH-stimulated ACTH production (Samson et al., 1995). In that same year, Parkes and May (1995) documented an ACTH-suppressive action of ADM when administered intravenously in conscious sheep, but no such effect on plasma concentration of ACTH was noted when ADM was administered intraventricularly. From both studies, it was obvious that ADM is involved in inhibiting ACTH release.

Taking these facts together and considering that in Cushing disease there is excessive secretion of ACTH, it may be that the associated increase in blood ADM levels described by Letizia et al. (2000) might induce a decrease in ACTH secretion, albeit an ineffective one. In this case, one could ask which step is being affected, as apparently an increase in ADM levels does not significantly reduce ACTH secretion. Circulating ADM is present, but perhaps its receptor system is inactive. More studies are needed to resolve these questions.

In contrast to the previously cited studies, Mimoto et al. (2001) reported that basal as well as CRH-stimulated ACTH secretion were not affected by co-incubation of cultured rat corticotrophs in ADM. Some authors (Taylor and Samson, 2004) even described an increase in plasma levels of ACTH after central administration of ADM.

As shown by our results, the nontumoral adeno-hypophysis displays strong ADM immunopositivity while blood levels of ADM (Letizia et al., 2000) are known to be relatively low in control patient. The lack of significant ADM release into the blood stream suggests that it exercises an autocrine or paracrine action within the normal adeno-hypophysis.

We found that ADM is synthesized in gonadotrophs in nontumoral adeno-hypophyses, in accordance with the results of Montuenga et al. (2000). In addition, our results regarding the ADM immunoreactivity in pituitary tumors are consistent with those of Knerr et al. (2001). These authors detected ADM mRNA in all pituitary tumor specimen investigated. In their studies ADM mRNA levels were slightly higher in prolactinomas than in somatotropinomas. The investigation of Knerr et al. (2001) also affirmed that hypoxia influences ADM production. For this reason, they refrained from using autopsy-obtained tissues and justified the lack of normal pituitary glands in their study. Nakayama et al. (1998) reported that hypoxia stimulates the accumulation of ADM mRNA in cultured human colorectal carcinoma cell line and immunoreactive ADM in the culture media. Garayoa et al. (2000) provided evidence that hypoxia upregulates ADM expression in several human tumor cell lines. In our study, tumor samples were biopsy-derived and the nontumoral adeno-hypophyses were biopsy and autopsy-obtained, we did not appreciate any difference in tissue ADM immunoreactivity. In the same article, Knerr et al. (2001) showed a relatively high ADM mRNA content in GH-producing adenomas and prolactinomas, a finding in contrast to our study in which such adenomas exhibited among the lowest levels of ADM immunopositivity (0.56 ± 0.19) along with those exhibited by other GH-containing tumors (mammo-

somatotroph and plurihormonal adenomas).

In summary, nontumoral and neoplastic pituitary tissues express ADM, immunopositivity being stronger in normal pituitaries than in adenomas. In nontumoral adenohypophysis, immunopositivity for ADM was mainly co-localized with FHS and LH in gonadotroph cells whereas in the other cell types the co-localization was mild. The action of ADM in the adenohypophysis is still controversial. Some studies have suggested that it decreases ACTH secretion, whereas others describe an opposite effect. More research will no doubt provide deeper insight into the role of ADM in the function of the adenomatous and nontumoral pituitaries.

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