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Sergio Vidal · Kalman Kovacs · Eva Horvath
Bernd W. Scheithauer · Takao Kuroki
Ricardo V. Lloyd

Microvessel density in pituitary adenomas and carcinomas

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Abstract Tumor growth depends on several factors, including angiogenesis. Tumors cannot grow if new vessels are not formed to supply the cells with oxygen and other nutrients and to remove waste products. Increased angiogenesis can be correlated with tumor growth and metastatic potential in many tumor types, indicating that neoformation of vessels is a prognostic indicator of tumor behavior. We evaluated microvessel densities in 157 various pituitary adenoma types and seven pituitary carcinomas using immunocytochemistry for CD-34 antigen, a reliable marker of endothelial cells. The lowest percentage of microvessel density was found in growth hormone-producing adenomas, the highest level in pituitary carcinomas. In general, no major correlation was found between MIB-1 index (an indicator of cell proliferation) and microvessel density. The statistical study also demonstrated no gender-dependent changes in the microvessel density of pituitary tumors. Although the microvessel density was not significantly different in relation to invasiveness of pituitary tumors, our results demonstrate a tendency of invasive pituitary tumors to be more highly vascularized than non-invasive ones. Dopamine agonist and long-acting somatostatin analog treatment compared with untreated tumors did not significantly affect microvessel densities. Statistical differences were demonstrated in the microvessel density of macroadenomas between patients older and patients younger than 40 years. Significant differences were also apparent in the microvessel densities between microadenomas and macroadenomas diagnosed in young patients but not in the older age group. The strongly positive correlation observed between microvessel density and age is consistent with the view that age of the host may have an influence on the extent of neovascularization of pituitary adenomas.

Keywords Angiogenesis · Immunohistochemistry · Pathology · Pituitary adenoma · Pituitary carcinoma

S. Vidal (✉)
Department of Anatomy, Campus Universitario de Lugo, Lugo, Spain
e-mail: svidal@lugo.usc.es
Tel.: +416-864-5858, Fax: +416-864-5870

S. Vidal · K. Kovacs · E. Horvath
Department of Laboratory Medicine, St. Michael's Hospital, University of Toronto, 30 Bond Street, Toronto, Ontario, M5B 1W8, Canada

B.W. Scheithauer · R.V. Lloyd
Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, Minnesota, USA

T. Kuroki
First Department of Neurosurgery, Toho University, School of Medicine, Tokyo, Japan

Introduction

Transformation from the normal to the neoplastic phenotype involves disruption of regulatory events underlying normal cell function. It is stated that the progression of malignant tumors is related to the expression of oncogenes and/or loss of tumor suppressor genes. In pathophysiologic terms, the function of such genes affects key processes, such as cell proliferation, apoptosis, angiogenesis, invasion, and metastasis. Angiogenesis, the formation of new blood vessels from preexisting ones, is of importance to the growth of solid tumors, which depend upon a vascular network for their nourishment and extension.

Tumor cells induce the formation of an extensive, albeit aberrant vascular network by the production and secretion of angiogenic factors, such as vascular endothelial growth factor (VEGF) and/or basic fibroblast growth factor (bFGF). The molecular mechanisms underlying the enhanced production of these factors are many-fold and include activation of oncogenes, such as ras [3, 36], inactivation of tumor suppressor genes, such as p53 [21, 37] and von Hippel-Lindau (VHL genes) [15, 34], activation of protein kinase C by tumor promoters [56], and cell stimulation by growth factors or cytokines [11] and hypoxia [38].

In contrast to the majority of solid tumors, Lloyd et al. [26] reported decreased expression of VEGF, the

most significant inducing factor of angiogenesis, in pituitary adenomas relative to the non-tumorous anterior pituitary. This observation correlates well with the subnormal microvessel densities reported in various pituitary adenoma subtypes and supports the proposition of Jugenburg et al. [18] that lack of significant angiogenesis underlies the slow pace of pituitary tumor growth and the rarity of metastases. The goals of the present study were to obtain a deeper insight into the role of angiogenesis in pituitary tumor growth, spread, and metastasis and to determine whether alterations in microvessel density play a role in tumor shrinkage induced by dopamine agonists and long-acting somatostatin analogs.

Materials and methods

Pituitary tumors (164 tumors), including adenomas and carcinomas, were selected from the Mayo Clinic tissue registry and from the consultation files of three of the authors (BWS, KK, and EH). All were obtained by means of transsphenoidal surgery. The patients included 75 men (mean age 52.9 years, range 11–81 years) and 89 women (mean age 45.7 years, range 17–80 years). The clinical stage of tumors in relation to size and invasiveness and proliferative activity and vascularization were evaluated for each case. These parameters are summarized in Table 1. Tumor size and invasiveness were evaluated on the basis of preoperative magnetic resonance imaging (MRI) scan and on operative findings. Tumors were classified as micro- or macroadenoma when 1 cm or less in dimension or greater than 1 cm, respectively. All specimens were promptly fixed in 10% buffered formalin, routinely processed, paraffin embedded, and cut at 5 µm. Each tumor was characterized by means of histology [hematoxylin and eosin (HE), periodic acid–Schiff (PAS), Gordon-Sweet reticulin method] and immunocytochemistry for pituitary hormones, using the labeled streptavidin-biotin peroxidase complex method. The latter included the complete spectrum of pituitary hormones: growth hormone (GH), prolactin (PRL), adrenocorticotropic hormone (ACTH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), thyrotropic hormone (TSH), and alpha subunit. The sources, dilutions, and clonality of these antibodies and control methods have been previously described elsewhere [22, 23]. Many tumors were glutaraldehyde-fixed, routinely processed, Epon-embedded, and further analyzed using transmission electron microscopy. Each case was evaluated and assigned to one of six categories: GH-producing adenomas (ten cases); PRL-producing adenomas (37 cases); ACTH-producing adenomas (nine cases with Nelson's syndrome and ten cases with Cushing's disease); TSH-producing adenomas (ten cases); and a group of clinically non-functioning adenomas (eight ACTH silent subtype 1, 13 silent subtype 3, ten female and ten male gonadotroph adenomas, and ten non-oncocytic and ten oncocytic null cell adenomas). Lastly, seven pituitary carcinomas were also studied. In addition, ten bromocriptine-treated, PRL-producing adenomas and ten octreotide-treated, GH-producing adenomas were also examined.

The proliferative activity of each tumor was evaluated with the MIB-1 immunostaining (Immunotech Inc, Westbrook Me.; monoclonal antibody, 1:400), using the streptavidin-biotin peroxidase complex method [17]. The MIB-1 labeling index was manually determined and expressed as percentage positive nuclei. Fields were randomly selected. A mean of 30 fields with approximately 100 cells per field were assessed in each case. Cells were considered positive when unequivocal nuclear staining could be identified.

Vascularization of pituitary tumors was determined immunocytochemically. Staining was directed against CD-34 (Dako, Glostrup, Denmark; monoclonal antibody), a sensitive marker of endothelial cells. Preliminary titration experiments determined the optimal working dilution to be 1:25. After routine deparaffinization, rehydration, and blocking of endogenous peroxidase activity, sections were pretreated for the purpose of antigen retrieval by means of microwaving in 0.1 mM sodium citrate buffer (pH 6.0), as previously described [50]. Subsequently, treated slides were incubated with antisera and then exposed to the streptavidin-biotin peroxidase complex. Diaminobenzidine served as the chromogen. The CD 34-immunostained sections were examined using a computer image analysis system (Microimage, Media Cybernetics, Silver Spring, Md.). Microvascular density or percentage of pituitary tumor occupied by vessels, excluding fibrotic areas, was determined by measuring their cumulative area in each field. These counts were performed using a Provis X70 microscope (Olympus) attached to an Olympus DP10 digital camera. Images were taken us-

Table 1 Clinical and pathologic parameters in 164 patients with pituitary tumors. The significant differences between MIB-1 index and microvessel density were determined using Kruskall–Wallis analysis of variance and the Mann-Whitney *U* test. *GH* growth hormone; *PRL* prolactin; *ACTH* adrenocorticotropic hormone; *TSH* thyrotropic hormone

	Invasiveness		Tumor size		Carcinoma
	Invasive	Non-invasive	Macroadenoma	Microadenoma	
Age at surgery (years)					
Mean±SEM	50.9±2.4	47.5±2.4	51.6±1.7	37.7±3.3	59.4±3.5
Range	17–80	11–81	17–80	11–81	49–69
Gender (%)					
Male	44.4	44.6	50.5	19	20
Female	55.6	55.4	49.5	81	80
MIB-1 index	2.8±0.7[b]	1.0±0.1[a, b]	1.9±0.4[b]	1.4±0.3[a, b]	15.0±4.0
Microvessel density	2.9±0.3	2.6±0.2[c]	2.7±0.2[c]	2.6±0.3[c]	4.1±0.9
Tumor type (%)					
GH-producing adenoma	40.0	60.0	90.0	10.0	
PRL-producing adenoma	51.8	48.2	74.1	25.9	
ACTH-producing adenoma	35.7	64.3	35.7	64.3	
TSH-producing adenoma	42.9	57.1	87.5	12.5	
Non-functioning adenoma	34.7	65.3	97.8	2.2	

[a] $P<0.05$ vs invasive adenoma [b] $P<0.001$ vs carcinoma [c] $P<0.05$ vs carcinoma

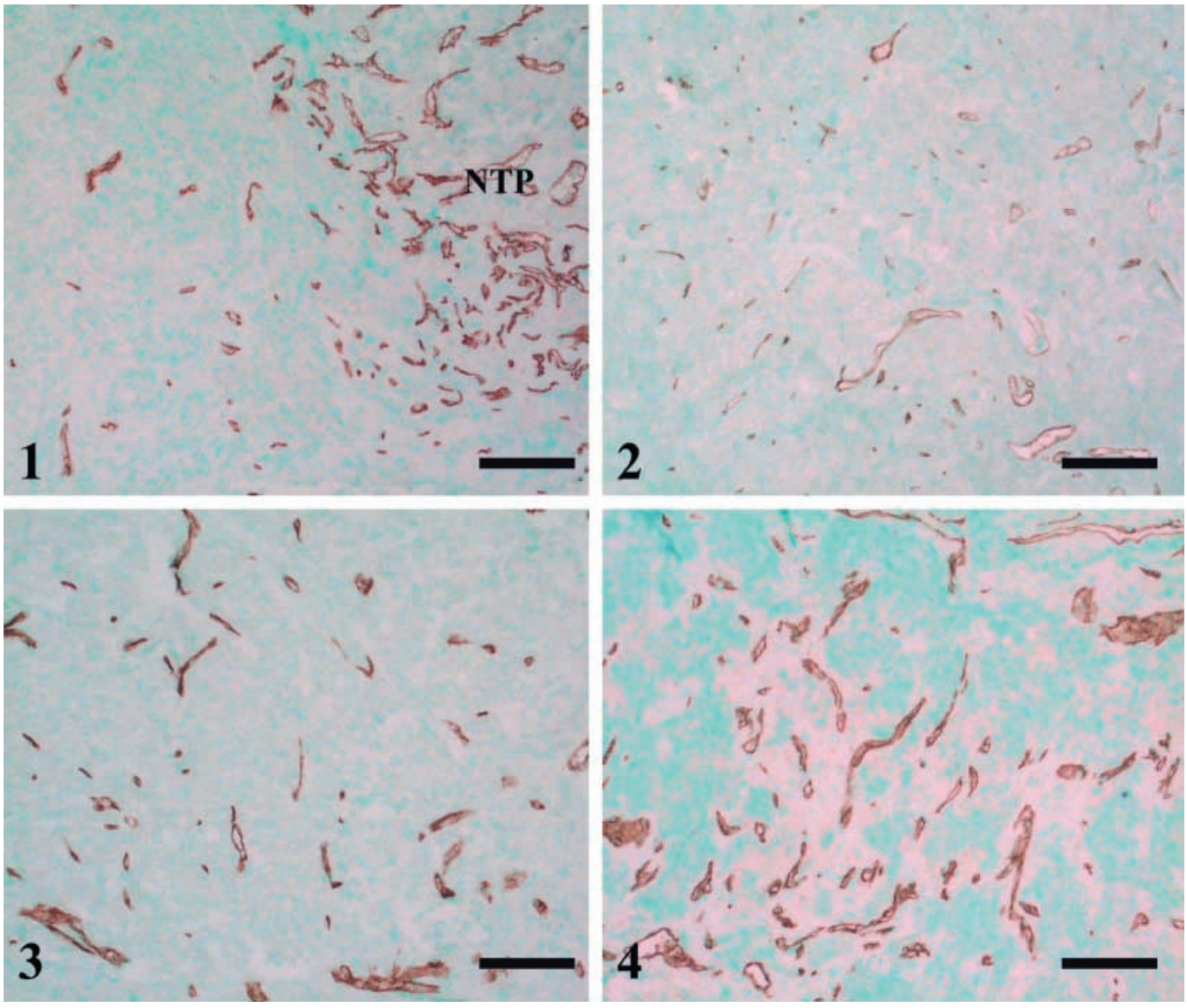

Fig. 1 Adenoma border. Compared with the non-tumorous adenohypophysis (NTP), microvessel density is decreased in the untreated growth hormone (GH) adenoma. Immunostaining for CD34. *Bar* 100 µm

Fig. 2 CD34 immunoreactivity in the octreotide-treated growth hormone (GH)-producing adenoma. *Bar* 100 µm

Fig. 3 Immunostaining shows CD34 immunoreactivity in the endothelial cells of the untreated prolactin (PRL) adenoma. *Bar* 100 µm

Fig. 4 Immunostaining for CD34 in pituitary carcinoma showing higher microvessel density than the other pituitary tumor types. *Bar* 100 µm

ing a 40× apochromate objective and were captured digitally at a resolution of 386 pixels. Prior to measurements of vessel count, each image was processed digitally to exaggerate the contrast between microvessels and their lumens. In each pituitary tumor, a total of 20 randomly selected fields (corresponding to 6.7 mm^2 of pituitary tissue) were assessed.

Data were tested for statistical significance using the SPSS statistical computer program (SPSS, Inc., Chicago, Ill.). Since assumptions for a parametric test were not valid (Kolmogorov-Sminov $P<0.05$), all data were evaluated using Kruskall–Wallis analysis of variance and the Mann-Whitney U test as a multiple comparison method. The Spearman test was used to assess the statistical significance of the correlation between tumor vascularity, patient age, and the MIB-1 labeling index. Differences of $P<0.05$ were considered statistically significant.

Results

In all tumors, MIB-1 immunostaining was detected in the nuclei of at least some neoplastic cells. Such immunoreactive nuclei were easily identified and quantified. MIB-1 labeling indices, the percentage of invasive tumors, and macro- and microadenomas in each pituitary tumor type are summarized in Table 1. In both normal pituitary and in pituitary tumors, only the vascular endothelium was immunoreactive for CD34. The immunopositivity was easy to quantify (Fig. 1, Fig. 2, Fig. 3, and Fig. 4).

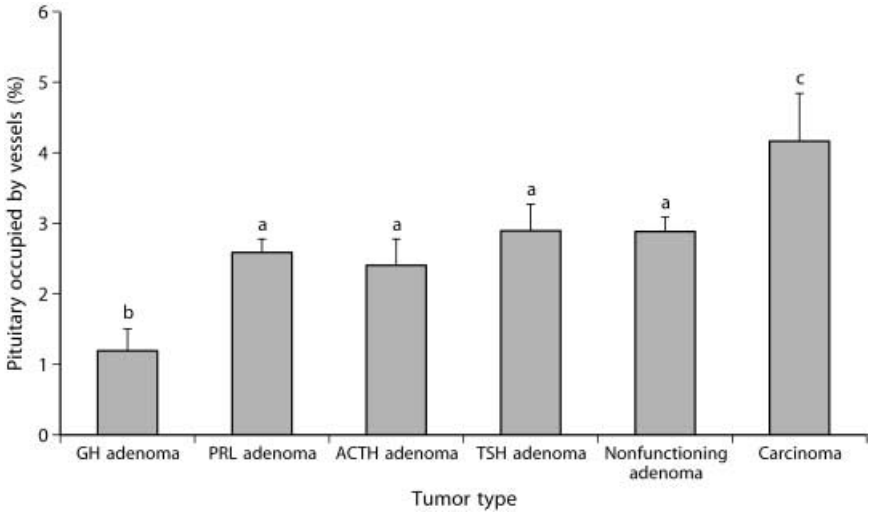

Fig. 5 Pituitary microvessel density in various pituitary tumor types. Data are expressed as percentage of pituitary occupied by vessels and represent the mean±SEM. Values with no letters in common are significantly different $P<0.05$ (statistical analysis with the Kruskall–Wallis analysis of variance and the Mann-Whitney *U* test)

The morphometric study demonstrated statistical differences in the microvessel density between the six categories of pituitary tumors (Fig. 5). GH-producing adenomas had the lowest microvessel density, whereas primary pituitary carcinomas were the most vascularized. It is of note that microvessel density was greater in pituitary carcinomas of PRL-producing and ACTH-producing type than in their corresponding pituitary adenomas. Only the microvascular density of the PRL-producing pituitary carcinomas was significantly higher ($P<0.05$) than that of the corresponding adenomas.

In contrast to GH-producing adenomas and pituitary carcinomas, PRL-producing adenomas, functioning ACTH adenomas, TSH-producing adenomas, and clinically non-functioning adenomas showed an intermediate degree of vascularization. Although no statistically significant differences were reported in the microvessel densities of these intermediately vascularized tumors, slight deviations were noted between each group (Fig. 5).

Considering all types of pituitary tumors, our statistical study demonstrated no firm correlation between MIB-1 labeling indices and microvessel density ($r=0.06$, $P=0.52$). Nonetheless, a positive correlation between these parameters was observed in two tumor types: pituitary oncocytic null cell adenomas ($r=0.70$, $P=0.04$) and the functioning ACTH adenomas of Cushing's disease ($r=0.74$, $P=0.02$). In contrast to the MIB-1 labeling index, strongly positive correlation was observed between microvessel density and age ($r=0.30$, $P=0.001$) in all tumors types. A similar correlation between these two parameters was noted in the female type of gonadotroph adenomas ($r=0.77$, $P=0.02$) and in TSH-producing adenomas ($r=0.78$, $P=0.01$).

The statistical study demonstrated no significant correlation between microvessel density of pituitary tumors and patient gender ($P=0.28$) or tumor invasiveness/non-invasiveness ($P=0.59$). Nonetheless, it should be noted that in 15.4% of invasive tumors, the microvessel density exceeded 5% (overall mean microvessel density 2.64%, range 0.40–9.01%), whereas values greater than 5% were observed only in 4.9% of non-invasive tumors. These results suggest a trend toward increased vascularity in invasive tumors.

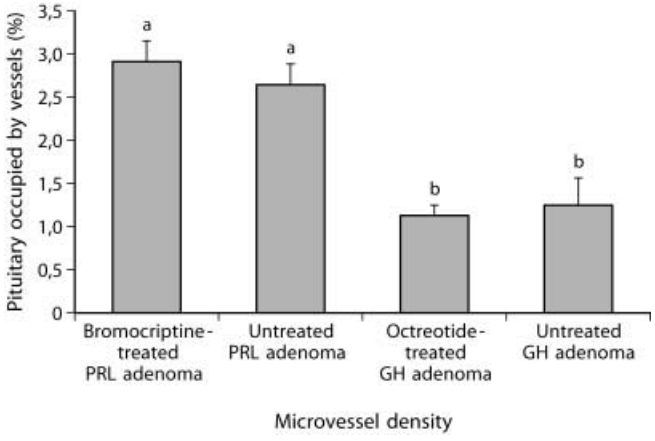

Fig. 6 Pituitary microvessel density in treated and untreated pituitary adenomas. Data are expressed as percentage of pituitary occupied by vessels and represent the mean±SEM. Values with no letters in common are significantly different $P<0.05$ (statistical analysis with the Kruskall–Wallis analysis of variance and the Mann-Whitney *U* test)

In relation to tumor size, 82% of all tumors were macroadenomas. Of these, 23% occurred in patients younger than 40 years and 77% in those older than 40 years. It is of note that significant differences ($P<0.001$) were demonstrated in the microvessel density of macroadenomas of young (1.68±0.20%) and older patients (3.13±0.21%). This observation did not correlate with differences between other parameters in the two groups, e.g., MIB-1 labeling index and invasiveness. In contrast to macroadenomas, no statistical differences ($P=0.31$) were noted in the microvessel density of microadenomas diagnosed in patients younger (2.34±0.39) and older (2.94±0.54) than 40 years of age. Whereas there were no significant differences ($P>0.09$) in the microves-

sel density of microadenomas and macroadenomas in patients over 40 years, significant differences ($P<0.05$) were noted in the younger age group.

As shown in Fig. 6, the mean microvessel density was only slightly increased in bromocriptine-treated PRL adenomas relative to untreated adenomas. However, no statistically significant differences were noted between these two adenoma groups ($P=0.33$). Similarly, no correlation was observed between microvessel density and MIB-1 labeling index in bromocriptine-treated and untreated PRL adenomas ($r=-0.06$, $P=0.72$).

In contrast to bromocriptine-treated PRL adenomas, octreotide-treated GH-producing adenomas tended to have lower micovessel densities than those untreated, although the differences ($P=0.66$) did not reach statistical significance (Fig. 6). In addition, no significant correlation ($r=0.07$, $P=0.81$) was noted between microvessel density and the decreased MIB-1 labeling indices in treated as opposed to untreated GH adenomas.

Discussion

Several studies have suggested that pituitary tumors are heterogeneous with a complicated, multifactorial etiology and pathogenesis [4]. Many pituitary tumors autonomously produce and secrete hormones, resulting in complex endocrinologic syndromes. The remainder are functionally "silent" and present as an expanding sellar mass, often associated with various degrees of hypopituitarism. Despite their complex clinicopathologic phenotypes, pituitary tumors share a number of features. All arise in adenohypophyseal cells and produce hormone-containing secretory granules. Most are slow growing, and metastases are exceptionally rare [24]. It has been shown that a large proportion of adenomas are invasive and spread to sellar/perisellar tissues. Such invasion, in itself, is not diagnostic of pituitary carcinoma. Defined as metastasizing tumors, they are exceedingly rare [35]. As in various other endocrine neoplasms, the histologic appearance of adenohypophyseal tumors is a poor predictor of aggressive behavior. Nonetheless, several studies have been undertaken to assess features predictive of invasiveness. In this regard, it has been demonstrated that clinically significant invasion is more frequent in macroadenomas than microadenomas [6]. Also, several candidates, such as matrix metalloproteinases, mitotic and MIB-1 labeling indices, p-27 and p-53 expression, and type IV collagenase production, have all been proposed as predictors of pituitary tumor invasiveness [2, 5, 19, 30, 33, 44, 45, 57]. In our studies, no significant difference was found in microvessel density between invasive and non-invasive pituitary tumors, although invasive pituitary tumors were slightly more vascularized than the non-invasive ones. Turner et al. [47] found significant differences in microvessel density between invasive and non-invasive PRL-producing adenomas but not in GH and ACTH adenomas. In our study, not every invasive adenoma had high microvessel density, indicating that in a subpopulation of invasive pituitary tumors, factors other than microvessel density underlie invasive potential.

Previous studies have demonstrated that angiogenesis, as assessed through microvessel density, is a significant prognostic factor in several tumor types [1, 7, 27, 32, 43, 46, 51, 54]. Neovascularization is crucial for tumor growth in that it facilitates tumoral oxygenation and nutrient perfusion and the removal of waste products [13, 20, 55]. Multiple parallel signaling pathways may be involved in the actions of mitogens that cause multiplication of tumor cells. In the current study, the proliferative potential of the tumor cells was examined immunohistochemically using the monoclonal antibody MIB-1, which recognizes Ki-67, a cell cycle-specific nuclear antigen. Ki-67 antigen is expressed throughout the cell cycle and reliably distinguishes proliferating from resting cells. Several publications have concluded that Ki-67, as detected by the MIB-1 antibody, is a useful marker of proliferative activity linked to tumor invasiveness and to the prognosis of pituitary tumors [44]. It has been suggested that every increase in neoplastic cells must be preceded by an increase in new capillaries converging upon the tumor [13, 20]. However, contrary findings have been reported also. Thus, there is no consensus on the prognostic significance of angiogenesis relative to tumor proliferation [1, 29, 31, 41]. Indeed, previous studies of different tumors, including pituitary adenomas [48], demonstrated no significant correlation between tumoral vascularity and proliferation and concluded that determination of microvessel density is of no use in predicting tumor growth. This is in agreement with our finding that no correlation exists between microvessel density and proliferative activity. Furthermore, since adenohypophyseal tumors are less vascularized than non-tumorous pituitary glands, it has been suggested that the lack of significant angiogenesis may underlie the slow pace of pituitary tumor growth [18, 49, 52]. In this case, a positive correlation should exist between angiogenesis and tumor cell proliferation. Our study provided no support to this notion. Several factors might contribute to the observed discrepancy between the lack of correlation regarding angiogenesis and tumor growth. One could speculate that growth of microadenomas (tumors smaller than 1 cm) does not require an extended microvessel network. In such small tumors, the original local blood supply and simple diffusion may be sufficient for the delivery of oxygen, other nutrients, and catabolite removal. This possibility is not supported by our finding, according to which no statistical differences were observed in microvessel density between microadenomas and macroadenomas in patients older than 40 years. Furthermore, in patients younger than 40 years, macroadenomas had significantly lower microvessel density than did microadenomas. Nonetheless, our observation of a significant change in microvessel density between macroadenomas of young and older patients may be related to differences in tumor behavior. Another possible explanation of a lack of tumor growth dependency upon angiogenesis might be a novel process recently described by Maniotis

and coworkers [28] in a study of uveal melanomas. These authors observed that in aggressive primary and metastatic melanomas, the tumor cells generate acellular microcirculatory channels composed of extracellular matrix and lined directly with tumor cells. Termed "vasculogenic mimicry" the process is not, strictly speaking a vasculogenic event, because true angiogenesis results in the formation of endothelial cell-lined vessels [12]. Vasculogenic mimicry has not been reported to occur in pituitary tumors, but this possibility can not simply be dismissed. Previous studies have described peliosis in pituitary tumors [8]. This rare process is characterized by the presence of multiple blood-filled spaces. Lastly, it is also possible that lack of significant angiogenesis in pituitary tumors may be a "protective mechanism" shielding the tumor from the effects of hypothalamic control mediated by releasing and inhibiting hormones. Consistent with this notion is the demonstration that pituitary tumor cells may function independently of hypothalamic control [40]. Indeed, several studies have strongly suggested that the development of pituitary tumors is related to an intrinsic pituitary cell defect, one leading to a monoclonal proliferation of tumor cells. The role of hypothalamic hormones in regulating the growth of such transformed cell clones remains a possibility [39, 40, 53] and deserves further study.

Previous studies of various types of tumors have shown that microvessel density is related to metastatic potential and may be an independent predictor of patient survival [14]. Metastasis of solid tumors is a complicated, multistep cascade that begins with invasion of capillaries. This being the case, tumor vascularization is clearly a requisite for metastasis, since poorly vascularized tumors would be compromised in their capacity for spread [14]. Consistent with the view that the occurrence of metastasis is dependent upon angiogenesis, we found that microvessel density provides valuable information regarding metastatic potential in pituitary tumors, microvessel density being higher in pituitary carcinomas than in adenomas.

In contrast to our previous observations in non-tumorous pituitaries [52], which showed no significant age-related changes in microvessel density in either sex, the present study found a strong positive correlation between tumoral microvessel density and patient age. This is not in agreement with the reported negative correlation between age and microvessel density in GH-secreting tumors [47]. Because angiogenesis is the result of a delicate balance between factors that stimulate or inhibit endothelial proliferation, several mechanisms might impact upon the behavior of vessels in the neoplastic and non-tumorous pituitary. Lloyd et al. [26] reported decreased expression of VEGF in pituitary adenomas relative to non-tumorous pituitaries. In addition, we demonstrated VHL protein, which represses VEGF transcription within adenomatous pituitary cells [51].

Lastly, since angiogenesis plays a pivotal role in tumor behavior, it is a potential target of anticancer treatment. The impact of chemotherapy and/or endocrine therapy upon angiogenesis is not well understood. Nonetheless, recent reports suggest that several compounds have antiangiogenic activity [10, 16]. The present study confirms previous observations that bromocriptine therapy does not influence significantly angiogenesis in prolactin cell adenomas [42, 47]. It is important to note that our finding of a slight increase in microvessel density in treated adenomas relative to untreated tumors, may be due to reduction of tumor cell size rather than some residual angiogenic effect of bromocriptine.

In spite of the prior demonstration that octreotide is an inhibitor of angiogenesis both in vitro and in vivo [9, 25], our study found slight but no significant decrease in microvessel density in octreotide-treated GH adenomas relative to untreated lesions. Clearly, more information is needed regarding the molecular mechanism underlying angiogenesis in pituitary tumors. Such understanding could contribute to the development of new antiangiogenic drugs useful in the treatment of pituitary tumors unresponsive to available medical therapies.

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