Pituitary (2013) 16:303–310
DOI 10.1007/s11102-012-0421-9

Enhanced nestin expression and small blood vessels in human pituitary adenomas

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Published online: 11 August 2012

Abstract The role of angiogenesis in human pituitary tumor progression is questioned. Our aim was to characterize the morphologic changes that occur in the vasculature of pituitary adenomas, in correlation with the expression of nestin, a protein found in endothelial cells of newly formed vessels of developing organs. We also evaluated the relation of angiogenic markers and nestin with Ki-67 index. Immunohistochemical studies were performed on paraffin embedded samples of 47 pituitary adenomas and six normal pituitaries. We determined microvessel density (number of CD31+ or CD34+ vessels per square millimetre), vascular area (cumulative area occupied by vessels), average vessel size, and further classified vessels as small (<100 μm^2) or large (>100 μm^2). We correlated the above parameters with nestin expression and Ki-67 index. Lower vascular area compared to normal tissue was found in adenomas ($p < 0.05$). Interestingly, pituitary adenomas had significantly more small vessels than control pituitaries ($p < 0.04$ for CD31 and CD34). In tumors many capillaries were positive for nestin, while scarce staining was detected in controls, so that nestin positive area was significantly higher in tumors. Furthermore, nestin area correlated positively with the % of small vessels. Ki-67 correlated neither with vascular area nor with nestin expression. In human pituitary tumors there was a predominance of small capillaries in correlation with increased expression of the progenitor marker nestin. We suggest that angiogenesis is an active process in these tumors, in spite of their low total vascular area when compared to normal pituitaries.

Keywords Tumorigenesis · Angiogenesis · Proliferation · Nestin

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Background

Angiogenesis, the development of new blood vessels from preexisting vasculature, is a crucial process in normal physiology and an important event in several diseases including cancer. Its critical role in tumor development was first demonstrated by Judah Folkman [1, 2] and later confirmed by a large body of research. This complex process has been evaluated in a variety of tumors of unrelated origins and in many cases has been correlated to aspects of tumor behavior such as recurrence, survival and poor prognosis [3–5].

Because endocrine organs are highly vascularized in their normal state, changes during neoplastic development may be quite different from those occurring in less vascularized tissues.

Pituitary adenomas growth necessarily bears angiogenesis within the tumor mass, nevertheless, the role of angiogenesis in pituitary tumor progression has been questioned. Differences in the angiogenic pattern of pituitary tumors have yielded highly controversial results concerning hormonal phenotypes, size or invasion [6]. Some data point to increased angiogenesis, while others have described that pituitary tumors are usually less vascularized than the normal pituitary tissue [6, 7].

Measurement of micro vascular density (MVD) and vascular area (% area occupied by vessels) have been used to investigate angiogenesis in different tumors. These parameters correlate with the expression of proangiogenic factors such as fibroblast growth factor-2 (FGF2) and vascular endothelial growth factor-A (VEGF) [8], and MVD has been shown to be closely related to tumor growth and metastasis in breast, colorectal, lung and urogenital cancers [9]. Both, MVD and vascular area, have been assessed by counting vessels labeled using immunohistochemistry with antibodies to different endothelial markers such as clusters of differentiation 31 and 34 (CD31 and CD34). An additional reliable marker of neovascularization is nestin. It is a class VI intermediate filament protein that participates in cytoskeleton formation and has been found in endothelial cells of newly formed blood vessels of developing organs [10]. In particular, it has been reported that nestin-containing cells in the pituitary gland play an important role in its cellular and morphological plasticity throughout life [11]. Moreover, nestin expression was detected in endothelial cells of pituitary adenomas and in a carcinoma sample [12], even though a precise role for the protein in these pathologies was not forwarded. In particular, the role of nestin in pituitary adenomas in relation to angiogenesis has not been addressed.

Therefore, the purpose of our work was to characterize the morphologic changes that occur in human pituitary adenomas in relation to angiogenesis. To this end, we determined the MVD, vascular area, and the size of vessels in a series of pituitary tumors of different immunotypes compared to normal pituitary specimens. Concurrently, we analyzed the expression levels and localization of nestin in the samples, and evaluated the relation of this progenitor cell marker with the vascular network that arises in the adenomas. Finally, we evaluated the correlation of angiogenic markers and nestin with the cell proliferation index evaluated by Ki-67 antigen immunodetection.

Methods

Patients

Forty-seven surgically obtained pituitary adenomas and six non-tumorous pituitaries were included in the study. Adenomas were previously classified according to hormone production, and the clinical, endocrine and radiological data were used to fully characterize each tumor type. There were 9 growth hormone (GH) and 11 prolactin (all resistant to dopamine agonist therapy) secreting tumors, and 27 non-functioning pituitary adenomas. Of the total of 47 adenomas, the MRI revealed that 80 % were invasive, classified as sellar or suprasellar macroadenomas with sphenoid sinus invasion, and compression or displacement of quiasm. The rest were non invasive microadenomas or intraselar macroadenomas. Dopamine resistance was defined as the failure to reach normal serum prolactin levels and/or reduce tumor volume in at least a 50 % [13]. In this cohort of samples we did not have surgical material of untreated prolactinomas.

Patients' age ranged from 19 to 79 years (mean 45 years ± 13.6); 30 were women (64 %).

Samples were immediately fixed after surgery in 10 % neutral buffered formalin, dehydrated in graded ethanol and embedded in paraffin. Sections of 4 μm thickness were cut and immunohistochemistry for different antigens was performed.

The project was approved by the Research Ethical Committees of the Instituto de Biología y Medicina Experimental-CONICET, and the Santa Lucía Hospital, Buenos Aires. Patients signed an approved informed consent.

Immunohistochemistry

Immunohistochemistry of paraffin embedded samples was perfomed as previously described [14, 15]. Tissues were exposed to the primary antibody over night at 4C. Antigen retrieval procedure was performed using citrate buffer and the microwave technique. Replacement of the primary antibody with phosphate buffer served as a negative control. Subsequently, slides were incubated with the appropriate secondary antibody and then with streptavidin/biotin peroxidase complex. Diaminobenzidine served as chromogen.

Microvessel density, vascular area, and vessel size assessment

Goat polyclonal anti-CD31 (sc-1506, 1:200, Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit polyclonal anti-CD34 (sc-9095, 1:200, Santa Cruz) were used. As a measure of angiogenesis we determined the MVD by counting the number of CD31+ or CD34+ vessels per square millimetre, and the vascular area by determining the cumulative area of the tumorous or normal glands occupied by CD31+ or CD34+ vessels (luminal area was quantified) in relation of the total area (% CD31+ or CD34+ area / total area). We also studied the size of vessels, and classified them as small (<100 μm^2) or large (>100 μm^2). A more detailed analysis was also performed determining the % of vessels with areas ranging from 50 to $>2{,}000$ μm^2. Images of randomly

selected fields were recorded using 40× or 100× objective, using a Zeiss Axiostar Plus microscope and a Canon PowerShot G6 digital camera. Three slides per pituitary were analysed and at least four images per slide at 400× of total magnification were counted by the image processing and analysis software: Image J, http://rsbweb.nih.gov/ij/.

Nestin measurement

Immunostaining of nestin was made with the mouse monoclonal nestin antibody (ab22035, 1:80, Abcam Cambridge MA). Immunopositivity was evaluated at high magnification (1000×) with Image J software. The nestin positive area (μm^2) stained with diaminobenzidine was quantified and normalized to the total area (25,500 μm^2), expressing the result as percentage.

Proliferation assessment

The antibody used was rabbit polyclonal anti-Ki-67 (sc-15402, 1:100, Santa Cruz Biotechnology). The Ki67 labeling index was manually determined by counting brown stained nuclei, and expressed as percentage of positive nuclei. A mean of 30 fields, were analysed at 400× of total magnification each containing approximately 100 cells. Cells considered positive showed unequivocal nuclear staining.

All parameters were determined independently by at least 2 persons, without knowledge of the sample type, and discordant cases were solved by simultaneous review. Finally the results were correlated with the age of the patient and the years since first diagnosis of pituitary adenoma (time from the detection of the tumor until surgery).

Statistical analysis

Since assumptions for a parametric test were not valid (Kolmogorov-Smirnov $p < 0.05$), the Kruskal-Wallis analysis of variance was used for between-group comparison of more than two groups. Post-hoc Dunn test was employed when necessary. Mann Whitney U test was used when only two groups were compared. Correlations were

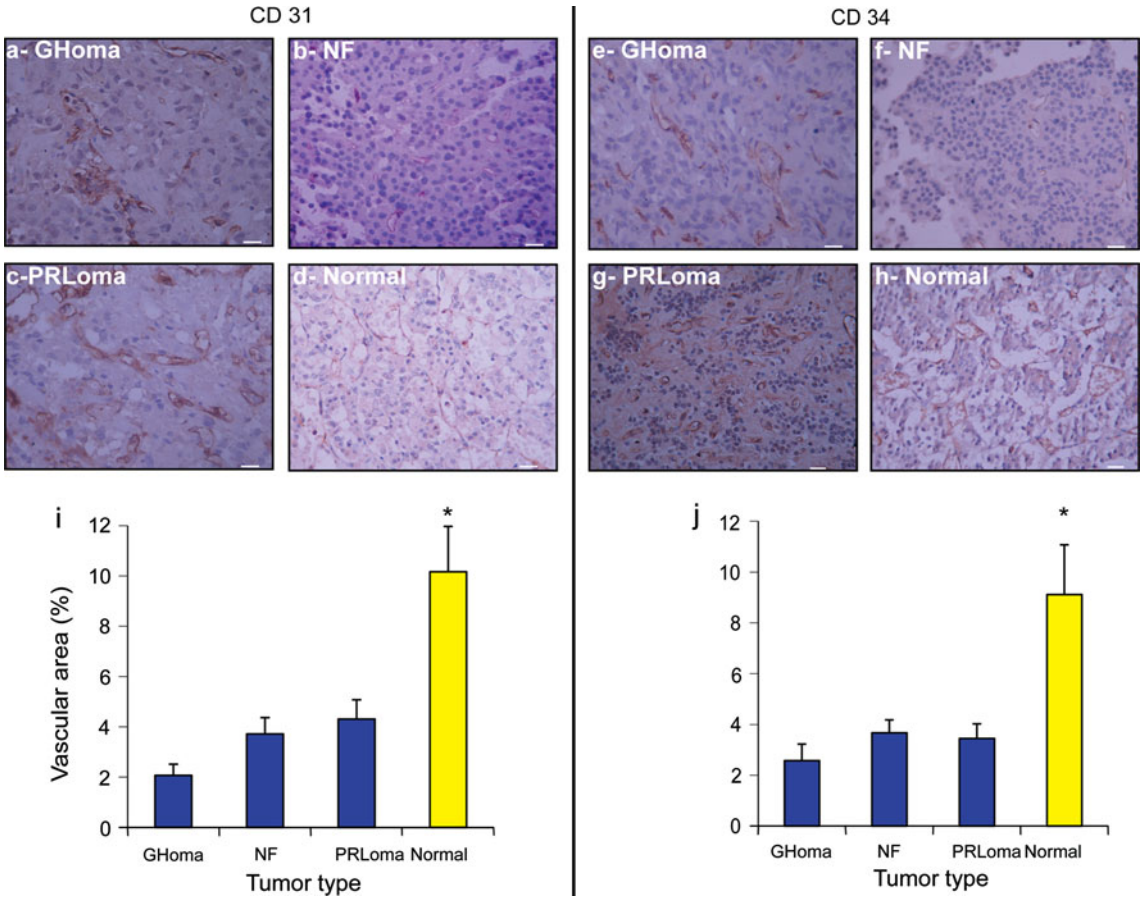

Fig. 1 Reduced vascular area in human pituitary adenomas. CD31 and CD34-stained blood vessels in GHoma (*a*, *e*), NF (*b*, *f*), PRLoma (*c*, *g*) and normal pituitary (*d*, *h*) sections, respectively. Nuclei were counterstained with hematoxylin dye. *Bar* 20 μm. *i* and *j* Vascular area was decreased in human pituitary adenomas compared to normal pituitaries determined by immunostaining for CD31 (N = 6, 17, 5, 6) and CD34 (N = 6, 20, 11, 6) for GHoma, NF, PRLoma and Normal pituitaries, respectively. * $p < 0.05$, control versus adenomas for both antibodies

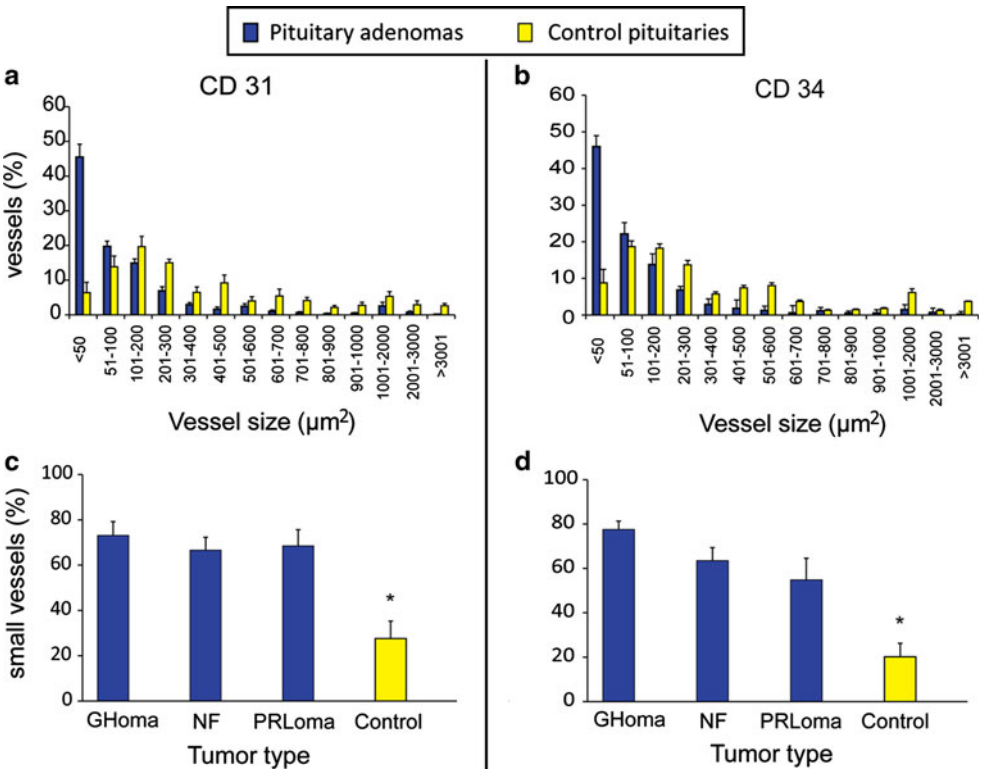

Fig. 2 Increased number of small vessels in pituitary adenomas. Distribution of CD31+ (**a**) and CD34+ vessels (**b**) according to their size. Normal pituitaries (N = 6) and pituitary adenomas (N = 28 and 37 for CD31 and CD34, respectively). Prevalence of small capillaries in human pituitary adenomas showed as the percentage of vessels with an area less than 100 μm^2, stained with CD31 (**c**) and with CD34 (**d**). * $p < 0.05$ normal glands versus adenomas. (N = 6, 17, 5, 5 for CD31 and 6,20,11,5 for CD34 for GHoma, NF, PRLoma and Normal pituitaries, respectively)

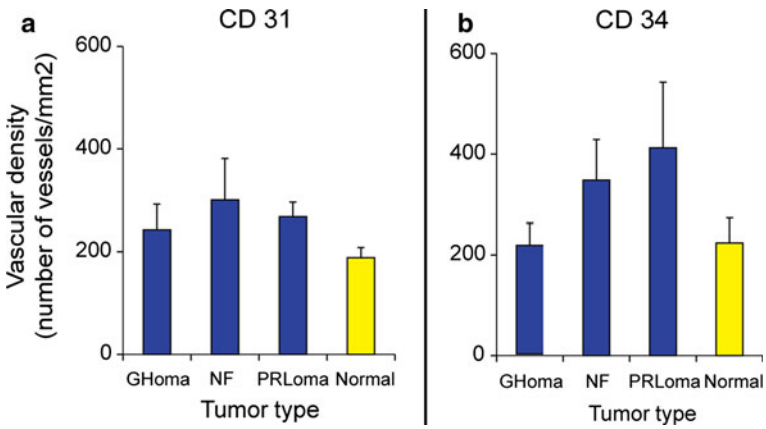

Fig. 3 Similar vascular density between tumoral and normal pituitaries. Microvessel density determined as the number of vessels/mm^2 for CD31 (**a**) (N = 6, 17, 5, 5) and CD34 labeling (**b**) (N = 6, 20, 11, 5) in GHoma, NF, PRLoma and Normal sections, respectively. No significant differences were found in the present series

performed by the Spearman test. Differences were considered significant if the p value was <0.05.

Results

Given the controversial data related to vascularity in pituitary tumor development, we performed immunohistochemistry labeling vessels with antibodies for the endothelial cell markers CD34 and CD31 on paraffin slides of normal and tumoral pituitary tissues. The antibodies used yielded unequivocal immunostaining of most of the microvessels present both in tumoral and normal pituitaries (Fig. 1a–h). Only endothelial cells were stained with either antibody, and results using both antibodies correlated significantly ($p < 0.01$, not shown).

In accordance with the high vascularization of normal endocrine glands, total vascular area was lower in adenomas (<3.5 and 4.5 % for CD34 or CD31, respectively, for all adenomas) when compared to normal pituitaries (7.5 and 14.5 % for CD34 and CD31, respectively; Fig. 1i, j, $p < 0.020$ for tumors vs. normal glands with both markers). We analyzed the distribution of vessels according to their size and found that pituitary adenomas had a higher

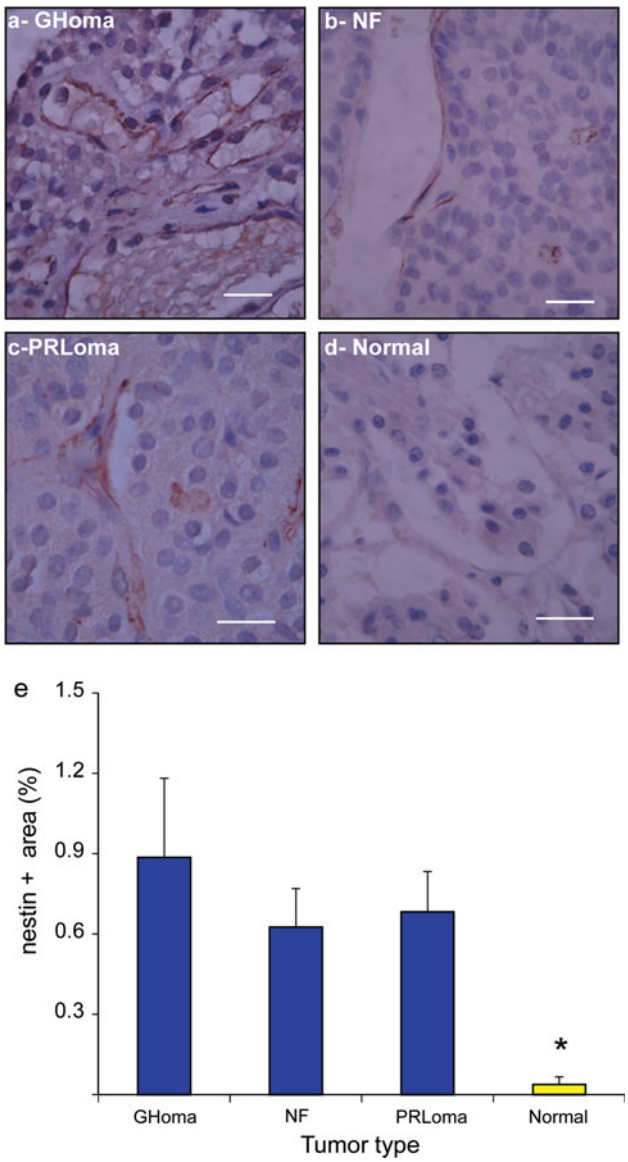

Fig. 4 Increased nestin expression in pituitary adenomas. Different patterns of nestin staining found in pituitary adenomas and normal pituitary sections. Incomplete stained endothelia of a Non functioning adenoma (**b**) and a PRLoma (**c**) where thin labeling in the perivascular zone is seen; or thick and almost fully marked capillaries are shown in the GHoma (**a**). **d** Normal human pituitary where nestin expression is almost absent. *Bar* 20 μm. **e** Percentage of nestin positive area in adenomas and normal pituitaries. At least 10 fields of ×1000 magnification were quantified for each sample. * $p < 0.05$, control versus all adenoma types. N = 6, 13, 7, 5 for GHoma, NF, PRLoma and normal pituitary, respectively

percentage of vessels ranging from 5 to 100 μm^2 compared to normal pituitaries (Fig. 2a, b). We therefore classified vessels as large (area >100 μm^2) or small (area <100 μm^2), and found that pituitary adenomas had significantly higher % of small vessels, than control pituitaries ($p < 0.02$ and 0.04 for CD31 and CD34 respectively, Fig. 2c, d). No differences between adenoma subtypes were found. On the other hand, MVD was not different in adenomas of different types, compared to control pituitaries (Fig. 3a, b, determined by CD31 and CD34, respectively), highlighting the fact that the predominant feature that may differentiate tumoral and normal pituitary vessels was their size and not their number.

We next determined nestin expression and observed that normal human pituitaries had scarce labeled cells (Fig. 4d), while all adenoma samples showed nestin expression which localized mainly around vessels and sometimes also as thin threads, free of an already conformed vascular bed (Fig. 4a–c). Many vessels were irregularly stained for nestin whereas in others the endothelium was completely marked by a continuous line. Immunopositivity was sometimes lined shaped and extremely thin, and in other cases a thicker labeling, not resembling the classical shape of endothelial cells was evidenced.

Quantitative analysis of nestin expression showed that nestin positive area was higher in adenomas than in controls, (Fig. 4e, $p < 0.010$ for control vs. adenomas), and not different between macro or microadenomas (not shown). Moreover, nestin positive area in tumor tissues correlated with the percentage of small blood vessels (<100 μm^2), while an inverse correlation was found between nestin area and the percentage of large vessels (>100 μm^2) for each sample (Fig. 5, $p = 0.01$; correlation for CD34 stained small vessels vs. nestin, and $p < 0.02$ for large vessels vs. nestin expression, similar results were obtained for CD31). Furthermore, there was an inverse correlation of nestin expression with years since first diagnosis of adenoma ($p < 0.03$, data not shown), and not with patients' age (data not shown NS).

Finally, no significant correlation was observed between the nestin+ or the vascular area and the nuclear Ki-67 index in the different pituitary adenomas (Fig. 6a, b, respectively), indicating that, even though neovascularization participates in the progression of these tumors, it is not the only or preponderant factor which promotes proliferation of pituitary adenomas.

Discussion

Angiogenesis plays an essential rol in tumorigenesis [1], especially in malignant diseases such as colon, breast and prostate cancer. Overall, these neoplasms are characterized by a high MVD [4, 16, 17]. On the contrary, in the field of pituitary adenomas results are controversial [6, 18]. Some data point to increased angiogenesis, while others describe that pituitary tumors are less vascularized than the normal pituitary gland [6, 7, 19]. Low vascularization is a peculiar situation for tumors despite their benign nature, as even premalignant lesions like precarcinomas of the cervix and breast have increased MVD [20, 21]. However, some

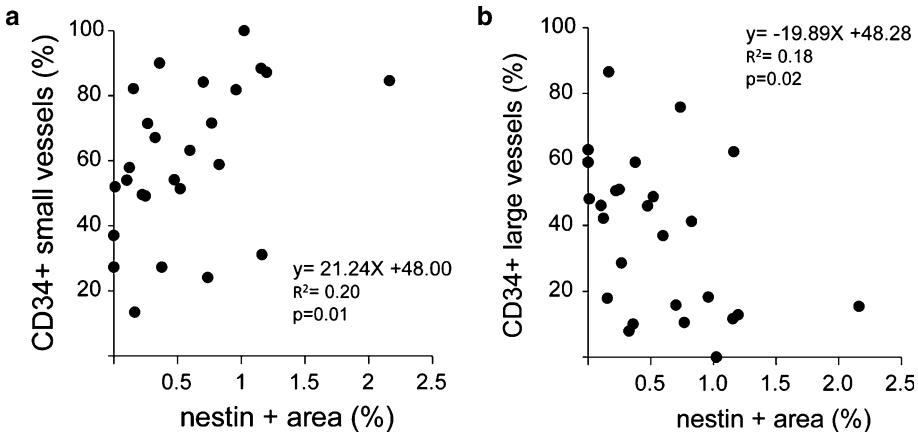

Fig. 5 Nestin expression correlates positively with percentage of small vessels. Spearman correlation for the % of nestin positive area with % of small (**a**) and large vessels (**b**) stained with CD34 antibody in human pituitary sections. A positive correlation was found for nestin and small vessels, and a negative one for nestin and large vessels. $p < 0.05$; N = 26. Similar results were obtained with CD31, not shown

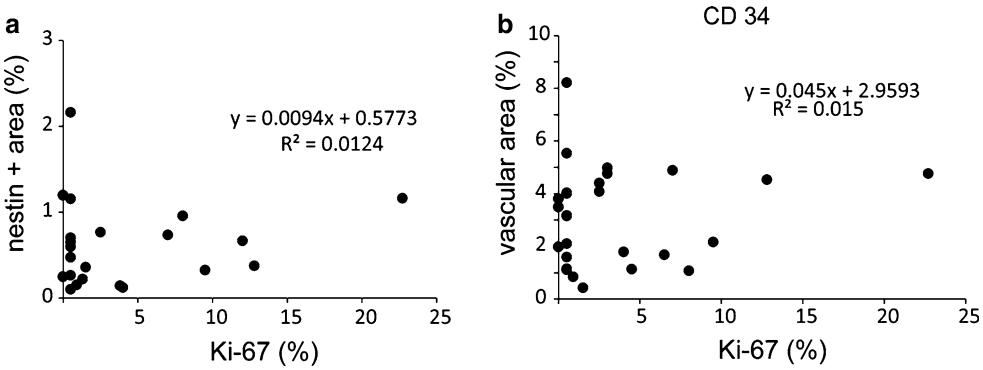

Fig. 6 Absence of correlation of Ki 67 index and CD34 or nestin staining. Spearman correlation for Ki 67 index with nestin positive (**a**) and CD34 positive (**b**) areas in human pituitary sections. $p > 0.05$; **a** N = 20 and **b** N = 26. Similar results were obtained with CD31, not shown

benign tumors that hardly ever progress to malignancy were reported with lower vascular density when compared to normal tissue [22–24]. We now show that indeed vascular area is lower in pituitary tumors compared to the normal gland, but that vessel sizes are markedly different in the normal and tumoral pituitary, and suggest that the increase % of small vessels in adenomas may be the predominant feature associated with angiogenesis. In accordance with our results, Itoh et al. [25] suggested that angiogenesis in the tumoral pituitary may occur with changes in diameter and shape of blood vessels.

In agreement with the involvement of angiogenesis in pituitary adenomas, we previously found increased VEGF expression in a cohort of dopamine resistant human prolactinomas, and a strong correlation of VEGF and CD31 expression in different pituitary adenoma types [26]. Furthermore, in an experimental model we demonstrated high VEGF expression in prolactinomas [14], and found that local and systemic antiangiogenic treatment of prolactinomas in *Drd2*$^{-/-}$ mice resulted in substantial tumor and prolactin inhibition [27]. In the systemic treatment, proliferation index was reduced when VEGF was blocked, pointing to the importance of angiogenesis in the growth of experimental resistant prolactinomas [27].

On the other hand, we show here that nestin expression was evidenced only in the adenomatous pituitaries, and correlated positively with the percentage of small vessels and negatively with years since first diagnosis of pituitary adenoma. Nestin was originally described as a neuronal stem/progenitor cell marker in cells of the developing central nervous system [28]. It was also detected in various neoplasms such as astrocytomas and malignant gliomas, including glioblastoma multiforme [29], and prostate cancer [30]. In these tumors nestin was generally expressed in immature endothelial cells generated in the course of angiogenesis [10, 31], and in the adult human pancreas nestin localized in endothelial cells predominantly of small caliber [32]. In our present results nestin localized mainly associated to blood vessels, and the inverse correlation of nestin with years since first diagnosis of pituitary adenoma or large blood vessels may suggest that nestin is expressed mainly in the setting of angiogenesis, and not in the quiescent endothelium, as previously suggested for other neoplasms [31, 33, 34]. Small vessels probably represent the newly formed blood vessels during pituitary adenoma generation. Indeed, nestin expression was evidenced only in newly formed capillaries growing into the infarcts and not in the necrotic capillaries, during pituitary infarction or apoplexy [35].

On the other hand, in the mouse anterior pituitary a side population enriched in stem-like cells expresses nestin [36, 37] and nestin has also been detected in rat pituitaries [38], where it did not colocalize with any of the pituitary hormones, and only sporadically with S100 protein of folliculo stellate cells. We now show a perivascular distribution of nestin immunoreactivity suggesting that this stem cell marker may be associated with endothelial cell development in pituitary adenomas.

A wide variation in staining patterns was evidenced between individual adenomas, even among ones belonging to the same immunotype, pointing to a complexity of the system. On the other hand, in the highly vascular normal pituitary gland, with mostly large blood vessels, the very low levels of nestin immunoreactivity would be in agreement with the quiescence of the endothelium of the mature vasculature.

We did not find any correlation between the proliferative index and angiogenic markers or nestin expression. Therefore, neither vascular area, nor progenitor cell expression, are direct indicators of the proliferative capacity of pituitary adenomas. To this respect, absence of correlation between Ki-67 index and angiogenic markers have been described in pituitary tumor development [6, 39–43]. These results indicate that the rate of proliferation in pituitary tumors is not directly related to neovascularization, and other factors may also affect the proliferation rate, invasiveness and behavior of tumors.

Conclusions

On the basis of our results which show a predominance of small capillaries and increased expression of the stem cell marker nestin in human pituitary adenomas, we conclude that angiogenesis is an active process in these tumors, in spite of their low total vascular area when compared to non tumoral pituitary. We believe that understanding the role of angiogenesis in the development of these tumors may facilitate therapeutical management in the cases of adenomas that cannot be controlled by conventional therapy.

Acknowledgments The authors acknowledge to the Consejo Nacional de Investigaciones Científicas y Técnicas, the Fundación Agencia Nacional de Promoción Científica y Técnica, and the Fundación Florencio Fiorini for the grants received that supported the work. This work was supported by the Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires, Argentina (CONICET, grant PIP 640, 2009, to Damasia Becú-Villalobos), Agencia Nacional de Promoción Científica y Técnica, Buenos Aires, Argentina (grant PICT N459, 2010, to Damasia Becú-Villalobos. Agencia Nacional de Promoción Científica y Técnica, Buenos Aires, Argentina grant PICT N1326, 2008 to Carolina Cristina, and Fundación Florencio Fiorini—Academia Nacional de Medicina, Buenos Aires, Argentina 2010 to Carolina Cristina).

Conflict of interest The authors do not have conflict of interests.

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