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ABSTRACT

Introduction and Objectives: angiogenesis is an essential process in tumor development. Nevertheless, discrepancies in the angiogenic pattern of pituitary tumors, in terms of hormonal phenotype, size or invasiveness have been found. Our aim was to study the expression of VEGF and FGF2 growth factors, and their importance in the vascularity of pituitary adenomas. We also quantified blood vessels with the endothelial cell markers CD31 and CD34 determining the vascular area, and the proliferation rate through PCNA and Ki67 index.

Materials and Methods: We studied 76 pituitary macroadenomas that were surgically resected in the period between 2006 and 2010 from a total of 276 patients with this pathology. Adenomas were classified into prolactinomas (PRL), somatotropinomas (GH), corticotropinomas (ACTH), non-functioning (NF) and plurihormonal (Ph) according to their hormonal secretion. Samples were collected in formalin, embedded in paraffin, and immunohistochemistry was performed from histological sections for endothelial markers CD31 and CD34; and for Ki-67 to study cell proliferation. VEGF, CD31 and PCNA were measured by Western blot. We compared results with normal glands (N=6).

Results: VEGF expression levels, found in all of the samples analyzed, were higher in resistant prolactinomas than in other pituitary adenomas. This protein was detected in endothelial cells of blood vessels and in tumor cells cytoplasm and nuclei. Fifty-six percent of samples were positive for FGF2, the other potent angiogenic factor studied, showing cytoplasmatic and extracellular matrix localization. We obtained a strong positive correlation between VEGF and CD31 in tumor samples, but we did not find lineal correlation between PCNA and VEGF, or between Ki-67 and VEGF in the samples studied. The vascular area was higher in normal tissues than in tumors when CD34 was used as endothelial cell marker.

Conclusion: The importance of studying angiogenesis in pituitary adenomas lies in the need to find molecular markers that can predict tumor behavior. We could demonstrate the expression of VEGF and FGF2, two potent angiogenic factors, and the existence of linear correlation between VEGF and CD31. Our results are indicative of the existence of angiogenesis in pituitary adenomas; therefore the blockage of angiogenesis might be proposed as an alternative strategy for cases of resistance to standard therapy. Rev Argent Endocrinol Metab 50:19-24, 2013

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Key words: pituitary, prolactinomas, neovascularization, vascular area
INTRODUCTION
Various theories have been proposed when studying the mechanisms of pituitary tumorigenesis: from genetic defects to hormone stimulation theories; however, a single theory cannot explain by itself the development of these tumors. It is not clear why most of these tumors are benign, what makes them become invasive, aggressive or resistant to dopamine receptors, as it is the case of some prolactinomas, or why only 0.5% produce metastases.

Angiogenesis, the formation of new blood vessels from preexisting ones, results from migration and proliferation of endothelial cells. It may be physiological or pathological, with the latter being and essential mechanism in the development of tumors (1, 2). The study of angiogenesis has allowed for the development of antiangiogenic therapies for various types of cancer.

The pituitary is a highly vascularized gland. This vascularity depends on the superior hypophyseal arteries branching from the internal carotid arteries. In turn, the meningohypophyseal trunk arises from the intracavernous carotid artery branching into the inferior hypophyseal arteries that drain into a pampiniform network, the portal system. The portal system envelopes the pituitary stalk and forms a vital link between the hypothalamus and the pituitary gland, which in turns communicates with a capillary network in the anterior lobe, which carries hypophysiotrophic factors into the pituitary and conveys anterior lobe hormones to the general circulation. Venous outflow of the pituitary is via collecting vessels that drain into the subhypophysial sinus, cavernous sinus, and superior circular sinus (3).

In pituitary adenomas, the role of angiogenesis is a controversial issue.

Discrepancies in the angiogenic pattern of pituitary tumors, in terms of hormonal phenotype, size or invasiveness have been found (4-6).

Angiogenesis is mediated by secretion of various growth factors, with the most important being: VEGF --vascular endothelial growth factor--, a potent mitogen for endothelial cells and inductor of vascular permeability; and FGF-2 --fibroblast growth factor 2—and its receptors. These factors, released by tumor cells, induce the development of new blood vessels to supply the necessary nutrients for tumor growth and are required for further dissemination of the tumor and metastasis.

Angiogenesis may be investigated by studying the expression of such factors, measuring microvessel density (MVD) and the vascular area, identifying and quantifying vessels with endothelial cell markers such as CD31 and CD34.

OBJECTIVES
In 2006, we initiated angiogenesis research with samples of human pituitary tumors with the aim of comparing findings with previous experimental studies where we had demonstrated that pituitary VEGF is increased in resistant prolactinomas of dopaminergic D2 receptor knockout mice when compared to wildtype mice, while FGF2 is decreased (7-9). Our aim was to study the importance of this process in the development of human pituitary tumors for application in the development of drugs capable of inhibiting this process, for therapeutic use.

MATERIALS AND METHODS
We studied 76 pituitary adenomas randomly selected from a total series of 276 that were surgically resected in the period between 2006 and 2010 at Hospital Santa Lucía and Clínica Santa Isabel. Patients signed an informed consent.

The endonasal transsphenoidal approach was used in 86% of cases. Image 1.
Adenomas were classified into prolactinomas (PRL), somatotropinomas (GH), non-functioning (NF), corticotropinomas (ACTH), and plurihormonal (Ph) according to their hormonal secretion. Table 1.

All prolactinomas used are resistant to dopaminergic agonist therapy. Criteria for defining resistance to this treatment include: failure to achieve normalization of PRL, a reduction to levels sufficient to achieve ovulation or less than 50% reduction in PRL levels. And as another goal of therapy is to achieve tumor shrinkage in order to reduce the mass effects caused by the tumor, resistance is also defined by a failure to achieve a 50% reduction in tumor size. As some patients achieve a good reduction in tumor size with no normalization of PRL levels and vice versa, we have adopted, as many other authors [10], the following definition of resistance to dopaminergic agonists: failure to achieve normal PRL levels and failure to achieve tumor size reduction of at least 50%.

Samples were collected in formalin, embedded in paraffin, and immunohistochemistry was performed from histological sections for endothelial markers CD31 and CD34; and for Ki-67 to study cell proliferation. When the size of tumor permitted, a second sample was prepared in Tris-EDTA buffer for western blot analysis of VEGF expression, PCNA and CD31 as previously reported [7,9].

Comparisons were made with normal glands (N = 6) BioChain Institute, Inc. USA.

RESULTS

All tumors showed VEGF expression. The level of expression of this angiogenic protein, determined by western blot, was significantly higher in resistant prolactinomas than in other pituitary adenomas (Graph 2).

By immunohistochemistry, this protein was detected in endothelial cells lining the blood vessels of adenomas as well as in tumor cells cytoplasms and nuclei.

In addition, only 56% of samples were positive for FGF2, showing mainly cytoplasmatic and extracellular matrix localization (Graph 3).

We determined the CD31 content by western blot analysis in tumor homogenates and even if we did not find significant differences in expression levels among the various types of adenomas, we found a strong positive correlation between VEGF and CD31 (Graph 4).

We determined the proliferation index by two markers: PCNA and Ki-67 and found no linear correlation between PCNA and VEGF or Ki-67 and VEGF in the samples studied (Graph 5).

We identified normal and tumor vasculature by immunohistochemistry for CD34 and determined the vascular area as area occupied by vessels / total area, which was higher in normal tissues than in tumors (Graph 6).
Image 1. a) Sagittal MRI of a sellar and suprasellar tumor b) Postoperative MRI showing a normal gland, c) surgical picture resecting a pituitary tumor d) total resection of the tumor, in the background there are residues of healthy gland.

TABLE 1. Classification of pituitary adenomas according to hormone secretion.
HSL: Hospital Santa Lucía, CSI: Clínica Santa Isabel

<table>
<thead>
<tr>
<th>Pituitary Adenomas</th>
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<th>CSI</th>
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<tbody>
<tr>
<td>Prolactinomas</td>
<td>13</td>
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<tr>
<td>Somatotropinomas</td>
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<tr>
<td>Non-functioning</td>
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<td>Corticotropinomas</td>
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<tr>
<td>Plurihormonal</td>
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Graph 2. VEGF expression levels/actin ratio expressed as percentage in relation to 100% taken for non-functioning adenomas. * P < 0.05 vs. ACTH, GH and NF; N = 4, 6, 23, 10 for ACTH, GH, NF and PRL, respectively.

Referencias de la figura:
VEGF/actina (%): VEGF/actin (%)
Tipo de tumor: type of tumor

Graph 3. Immunohistochemistry for FGF2 in ACTH-secreting adenoma with cytoplasmatic and extracelluar matrix location (left) and NF adenoma (right) with cytoplasmatic marker.
**Graph 4.** Linear correlation between VEGF and CD31 (measured by western blot for the group of pituitary adenomas), N = 29. The p shown corresponds to the Spearman correlation test.

Referencias de la figura:
VEGF/actina (%): VEGF/actin (%)
CD31/actina: CD31/actin

**Graph 5.** Association between VEGF and Ki67 cell proliferation indexes determined by immunohistochemistry (a) and PCNA quantified by Western blot (b) in specimens of pituitary adenomas, N = 42. NS.

Referencias de la figura:
VEGF/actina (%): VEGF/actin %
PCNA/actina (%): PCNA/actin (%)

**Graph 6.** Vascular area (area occupied by vessels / total area) expressed as percentage, determined by CD34 marker: N = 6, 18, 10 and 5 in GH, NF, PRL and Normal * P < 0.05 for normal vs. tumors)

Referencias de la figura:
Area vascular (%): Vascular Area
Tipo de tumor: type of tumor
DISCUSSION

Angiogenesis is one of the necessary and undeniable mechanisms of tumor formation, responsible for oxygenation, nutrient supply and development of metastasis. However, in the study of pituitary tumors, the fact that vascularity is higher in the normal gland than in adenomas (as we have shown in this study), and that these tumors very rarely metastasize, raised some doubts about the role of this mechanism in pituitary tumorigenesis.

In previous studies in experimental models, angiogenesis was shown to play an important role in the formation of pituitary adenomas (7-9). Accordingly, in vivo treatments performed with anti-VEGF therapy resulted in an inhibition of tumor growth and of prolactin synthesis in the pituitary gland of D2 dopamine receptor knockout mice (11). Based on these results, we studied the expression of angiogenic markers in human pituitary tumors.

Publications on angiogenesis of pituitary adenomas show controversial results. Some authors have found a higher expression of VEGF in normal pituitary glands when compared with adenomas, and a higher expression in carcinomas (12) but others have not found differences among the various pituitary adenomas (13). Our studies showed the presence of growth factors in all samples analyzed, with a higher VEGF expression in prolactinomas as compared to other tumors, and with FGF2 expression in 56% of the cases studied. Furthermore, the correlation between VEGF and vasculature markers is indicative of the development of angiogenesis in all types of pituitary tumors.

In addition, the lack of correlation between VEGF and cell proliferation markers indicates that the aggressiveness or proliferative capacity of the tumor are not limited by the degree of vascularity achieved by such tumor, and that other factors, such as primary genetic abnormalities, would be directly responsible for aggressiveness.

CONCLUSION

The study of angiogenesis in pituitary adenomas enabled us to demonstrate the participation of growth factors such as VEGF in the development of this type of tumors, analyze the tumor vasculature features evidenced by CD31 and CD34 markers and correlate expression levels of angiogenic proteins with the proliferation index measured by PCNA and Ki-67.

Our results as a whole are indicative of the existence of angiogenesis in pituitary adenomas; therefore the blockage of angiogenesis might be proposed as a complement to therapies directly aimed at stopping cell proliferation, as an alternative strategy for cases of resistance to standard therapy.
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