Prolactinomas: Role of VEGF, FGF-2 and CD31

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Abstract

Pituitary tumors rarely produce metastasis, but cause considerable morbidity and mortality. Each pituitary tumor of clonal origin represents the multifactorial result of failure of different regulatory events where growth and angiogenic factors may play critical roles in hormone secretion and cell proliferation. Prolactinomas, pituitary tumors which secrete prolactin, are generally treated successfully with dopamine agonists, even though a 10–15 % are resistant to this pharmacological therapy.

The role of angiogenesis in pituitary tumor development has been questioned, as pituitary tumors have been usually found to be less vascularized than the normal pituitary tissue. Nevertheless, a significantly higher degree of vasculature has been shown in invasive pituitary prolactinomas when compared to noninvasive prolactinomas. Furthermore, it has also been described that macroprolactinomas are more vascular than microprolactinomas.

Many growth factors and their receptors are involved in pituitary tumor development. For example, VEGF, FGF-2, FGFR1 and PTTG, which give a particular vascular phenotype, are modified in pituitary adenomas. Inhibitors of angiogenesis, Thrombospondin-1 and FGF-2 endogenous antisense have also been detected. In particular, vascular endothelial growth factor (VEGF) the central mediator of angiogenesis in endocrine glands, was encountered in experimental and human pituitary tumors at different levels of expression, and in particular,

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in dopamine resistant prolactinomas. Even though the role of angiogenesis in pituitary adenomas is contentious, VEGF, making permeable pituitary endothelia, might contribute to adequate temporal vascular supply and mechanisms other than endothelial cell proliferation. The study of angiogenic factor expression in aggressive prolactinomas with resistance to dopamine agonists will yield important data in the search of therapeutical alternatives.

Introduction

Pituitary Tumors

Pituitary tumors rarely produce metastasis, but cause considerable morbidity and mortality. In general, they result from monoclonal growth and intrinsic genetic defects which are related to oncogenes, suppressor genes, and genes responsible for differentiation. On the other hand, growth factors of hypothalamic or pituitary origin may act on aberrant cells, contributing to their proliferation (Ezzat 2001). Point mutations identified up till now can only account for a small percentage of pituitary tumors, and the mechanism of pituitary tumorigenesis is still unraveling.

Prolactinomas

Prolactin secreting adenomas are the most frequent type among pituitary tumors. Patients with prolactinoma usually present endocrinological symptoms resulting from hyperprolactinemia and, less commonly, with visual defects due to compression of the optic chiasm. Macroprolactinomas are benign, slowly proliferating tumors, although they may be locally highly aggressive, particularly in males, and invade adjacent structures. Giant prolactinomas (tumor volume exceeding 4 cm in diameter, and/or with prolactin levels higher than 3,000 ng/ml and mass effect) are a rare subcategory of macroprolactinomas, remain one of the greatest challenges in neurosurgery. Because of invasive growth, giant adenomas can compress or destroy adjacent structures, resulting in neurological dysfunction, and cavernous sinus compression. Pharmacological therapy with dopamine agonists remains the mainstay of treatment. This therapy is effective in >85 % of patients with prolactin-secreting pituitary tumors. A minority of patients show no primary response to either bromocriptine or cabergoline (Molitch 2005), and the development of dopamine agonist resistance in an initially responsive prolactinoma is unusual.

Angiogenesis in Pituitary Tumors

The formation of new blood vessels within neoplasms, termed angiogenesis, provides the tumor tissues with oxygen and basic energetic compounds. An increase in tumor size necessarily requires a corresponding increase in vascularization that is assured by means of the complex dynamic process of angiogenesis. In most human tumors, including breast, bladder, and stomach, angiogenesis has been shown to be correlated with tumor behavior. On the other hand, pituitary tumors are usually less vascularized than the normal pituitary tissue, as suggested by Schechter (Schechter 1972), and later confirmed by other authors (Jugenburg et al. 1995; Turner et al. 2000b). Differences in the angiogenic pattern of pituitary adenomas have yielded highly controversial results concerning hormonal phenotypes, size or invasion. In most studies, immunohistochemistry evaluation of different markers of microvascular density (MVD) such as cluster differentiation molecules (CD 31 and CD 34), Factor VIII (factor eight-related antigen), and ulex europaeus agglutinin I have been used. Nevertheless, the appraisal of MVD by immunohistochemistry has a number of substantial limitations, which are mainly due to the complex biology of tumor vasculature, and the irregular geometry of the vascular system (Vidal et al. 2003).

Some data point to increased angiogenesis in pituitary adenomas. For example, it has been described that macroprolactinomas are significantly more vascular than microprolactinomas (Jugenburg et al. 1995), and Turner et al. (2000a) demonstrated a significantly higher degree of

vasculature of invasive pituitary prolactinomas. Inhibitors of angiogenesis were effective in the suppression of growth of experimental prolactinomas and in angiographic studies the presence of additional arteries (which were not part of the portal system) were found in 66 % of patients with pituitary adenomas (Schechter et al. 1988). Nevertheless, the role of angiogenesis in pituitary tumor development has been questioned, as the normal pituitary is a highly vascularized gland.

Vascular Endothelial Growth Factor

Experiments over the past decades indicate that vascular endothelial growth factor-A (VEGF-A or VEGF) is a central regulator of angiogenesis in endocrine glands. VEGF-A is the founding member of a family of closely related cytokines that exert critical functions in vasculogenesis and in both pathologic and physiologic angiogenesis and lymphangiogenesis. The VEGF-A gene is located on the short arm of chromosome 6 and is differentially spliced to yield several different isoforms, the three most prominent of which encode polypeptides of 189, 165, and 121 amino acids in human cells. The protein has a hydrophobic leader sequence, typical of secreted proteins. It was discovered in the late 1970s as a tumor-secreted protein that potently increased microvascular permeability to plasma proteins. We can summarize its unique properties:

1. It is essential for normal developmental vasculogenesis and angiogenesis, as both null (*VEGF-A*$^{-/-}$) and heterozygote (*VEGF-A*$^{+/-}$) animals are embryonic lethals.
2. It increases vascular permeability to plasma and plasma proteins, a characteristic trait of the tumor microvasculature and a critical early step in tumor stroma generation.
3. It is a selective mitogen for vascular endothelium because its major tyrosine kinase receptors are selectively (though not exclusively) expressed on vascular endothelium.
4. It is overexpressed in a variety of human cancer cells (in human vascular tumors, including brain, colon, gastrointestinal tract, ovary, breast, and others).
5. It has a potential for evaluating prognosis in individual patients and as a therapeutic target.

Vascular Endothelial Growth Factor in the Pituitary Gland

VEGF expression has been described in all cell types in the normal pituitary, with greater expression in somatotroph and follicle-stellate cells. Using immunohistochemistry higher VEGF expression has been shown in the normal gland compared with adenomas (Lloyd et al. 1999), while the opposite has also been published. In a group of pituitary adenomas, ACTH and GH secreting adenomas, pituitary carcinomas had the strongest VEGF immunoreactivity (Lloyd et al. 1999). On the other hand, Viacava et al. (2003) found no differences in VEGF expression among tumors of different histotype, and McCabe et al. (2002) comparing VEGF in a series of adenomas composed of 77 % non functioning adenomas, and only 4 % of prolactinomas, found highest expression in nonfunctioning adenomas and GH producing adenomas. Elevated serum VEGF concentrations have been demonstrated in patients harboring pituitary tumors, and approximately 90 % of human pituitary tumors cultured in vitro show measurable VEGF secretion.

Using Western blot analysis of pituitary adenomas we found that VEGF protein expression was higher in prolactinomas compared to nonfunctioning (NF), GH, and ACTH secreting adenomas (Cristina et al. 2010). This finding may be related to the high percentage of macroprolactinomas in the series studied (11/12). In this respect, using angiogenic markers, it has been described that macroprolactinomas are significantly more vascularized than microprolactinomas. Furthermore, lower VEGF found in ACTH-producing adenomas may be consistent with the finding that VEGF production can be suppressed by glucocorticoids which are potent inhibitors of VEGF production in vitro (Lohrer et al. 2001). On the other hand, pituitary adenoma VEGF expression was similar in both sexes and was not influenced by age or years of adenoma evolution, when all adenomas were considered. This is in agreement with most studies which reveal that sex,

age or even rate of recurrence did not influence VEGF expression in pituitary tumors.

These data indicate that even though the role of angiogenesis in pituitary adenomas is contentious, VEGF might contribute to adequate temporal vascular supply with mechanisms other than endothelial cell proliferation. Tumor angiogenesis in the pituitary, as well as in other endocrine neoplasms, probably reflects the basic observation that tumors require neovascularization to grow; however, the changes that occur may be somewhat different from some other tissues that are less highly vascularized in the nonneoplastic state. Some data suggest that VEGF may prolong cell survival by inducing expression of the anti-apoptotic protein bcl-2 in pituitary adenomas, suggesting that part of its angiogenic activity is related to protection of endothelial cells from apoptosis. VEGF has been associated to intratumoral hemorrhage (Arita et al. 2004), and might also participate in the occurrence of pituitary peliosis, a form of vasculogenic mimicry. Peliosis may be linked to the permeabilizing function of this growth factor, and to the increased fenestration induced in blood vessels stimulated by VEGF overexpression. Peliosis occurrence has been related to high VEGF expression in hepatocarcinogenesis, spleen damage, and in a lethal hepatic syndrome in mice. This process may be seen in prolactinomas and other pitutitary adenomas, though it usually goes unrecognized. In dopamine D2 receptor knockout (*Drd2*$^{-/-}$) mice which develop lactotroph hyperplasia and eventually prolactinomas, we have described increased peliosis occurrence in these pituitary tumors in association with increased VEGF expression (Cristina et al. 2005).

Fibroblast Growth Factor-2

Basic fibroblast growth factor-2 (basic FGF, or FGF2), a potent angiogenic factor, was originally isolated from the bovine pituitary and has a pleiotropic activity affecting both vasculature and parenchyma cell proliferation and differentiation. It belongs to a large family of heparin-binding growth factors comprising at least 22 structurally related members. FGF2 expression is complex; at least four FGF2 isoforms (18, 22, 22.5, and 24 kDa) in human, and three (18, 21, and 22 kDa) in mouse are synthesized through alternative translation initiation from CUG codons. The 18 kDa isoform is predominantly cytoplasmic but can also be found in the extracellular matrix, while the higher-molecular-weight isoforms are localized in nuclei and ribosomes. The 18 kDa FGF2 isoform is highly expressed in the normal human pituitary, while pituitary adenomas produce predominantly the 24 kDa form. Recently, a 34 kDa isoform was reported with the most upstream CUG codon among all FGF2 forms. None of the isoforms have a typical secretory signal sequence, but alternative pathways have been described for their export from the cell. The biological effects of FGF2 are mediated through four high-affinity transmembrane receptors (FGFR1 – FGFR4) that have intrinsic tyrosine kinase activity. They can be found on a wide variety of cell membrane surfaces including endothelial cells where FGF2 exerts its proangiogenic functions.

Fibroblast Growth Factor-2 and FGFR1 in the Pituitary

FGF2 participates in pituitary development and proliferation and regulates hormone synthesis and secretion, affecting prolactin and TSH production. It is mainly produced by folliculostellate cells (FS) (Ferrara et al. 1987), although somatotrophs and gonadotrophs have also been reported to be sources of this growth factor. FGF2 participates in estradiol-mediated prolactinoma induction in rats under both physiological and pharmacological conditions. In the hyperplastic pituitaries of *Drd2*$^{-/-}$ mice, it induces prolactin secretion and cellular proliferation, and, interestingly has a differential subcellular distribution compared to that of wild-type pituitaries, which could be associated with different biological roles of this angiogenic factor in both genotypes (Cristina et al. 2007a). FGF2 is also expressed by human pituitary adenoma cells in vitro, and high levels of serum FGF2 were found in patients bearing pituitary tumors, declining following surgical adenomectomy. In the case of a giant invasive prolactinoma with loss of

response to dopamine agonist therapy we have recently reported strong immunoreactivity for both angiogenic factors VEGF and FGF2, as well as immunoreactivity for the endothelial cell marker CD31 indicating high vascularization of the adenoma (Mallea-Gil et al. 2009).

FGFR1 is found in the normal human pituitary as well as in pituitary adenomas, and its mRNA was described in the rat neural and anterior lobe. Furthermore, FGFR1 has been proposed as a candidate marker of pituitary tumors together with FGF2 and pituitary tumor transforming gene (PTTG); indeed, the FGF2 receptor FGFR1 was found to be highly expressed in pituitary tumors compared to the normal gland (McCabe et al. 2003). Furthermore, significantly increased FGFR1 mRNA expression was described in functioning tumors that invaded the sphenoid bone compared with those that did not, thus raising the possibility of using the FGFR1 as a molecular marker of tumor biological behavior (McCabe et al. 2003). On the other hand, it has also been determined that cytoplasmic FGFR1 immunoreactivity was inversely correlated with maximum pituitary tumor diameter.

Proteins and Genes Related to FGF2 in Prolactinomas

FGF4: DNA derived from human prolactinomas expresses transforming activity in heterologous cells and has sequences in close resemblance with those of *hst* gene. Overexpression of *hst* gene leads to increased production of FGF4. Shimon et al. (1996) demonstrated the function of the *hst* gene in rat lactotroph tumor formation and prolactin secretion. They were able to show that lactotrophs in 5 of 14 prolactinomas stained strongly with anti-FGF-4 monoclonal antibodies. Immunoreactive hst product in adenoma cells was observed in invasive prolactinomas. These findings imply a role of *hst* gene, and its product FGF4, in cellular proliferation, growth and aggressive behavior in prolactinomas.

PTTG: Estrogen promotes experimental prolactinoma development via induction of a pituitary tumour transforming gene (*pttg*) (Cristina et al. 2007b) that is located on chromosome 5q33. PTTG has been shown to be tumorigenic in vivo, by regulating basic fibroblast growth factor (FGF2) secretion and inhibiting chromatid separation.

Thrombospondin-1: (TSP-1) is a modular glycoprotein secreted by different cell types, including endothelial cells. It is composed of multiple active domains that bind to soluble factors, cell receptors, and extracellular components. TSP-1 was the first endogenous inhibitor of angiogenesis to be identified and its effect is due, at least in part, to its capacity to bind FGF2. TSP-1 agonists can inhibit experimental prolactinoma development (Sarkar et al. 2007).

FGF2 Endogenous Antisense (GFG) RNA: In *Xenopus laevis* oocytes, a 1.5 kb *FGF2* antisense (GFG) RNA complementary to the third exon and 3′UTR of FGF-2 mRNA has been implicated in FGF2 mRNA regulation. The human homolog has been localized to the same chromosomal site as FGF2 (chromosome 4, JO4513 adjacent to D4S430), confirming this as a human endogenous anti-sense gene. This GFG anti-sense gene also encodes a 35 kDa protein, and regulates cell proliferation and hormone secretion. Pituitary tumors have been shown to express FGF2 and GFG while the normal human pituitary expresses GFG but not FGF2; GFG protein levels are higher in the normal gland than in most tumors. Aggressive pituitary adenomas appear to express more FGF-2 than GFG mRNA (Ezzat 2001).

Truncated FGFR4: Altered FGF receptor expression has been found in pituitary adenomas (Ezzat 2001), and FGFR4 undergoes alternative transcription initiation in pituitary adenomas, giving rise to an oncogenic protein in pituitary adenomas of various subtypes. Expression of this pituitary tumor-derived (ptd)-FGFR4 protein is more frequent in macroadenomas than in microadenomas and correlates with the Ki-67 labeling index. Recent data suggest that ptd-FGFR4 alters cell adhesion by a mechanism that explains the loss of reticulin, which is the hallmark of pituitary adenomas. Taken together, these data suggest that deregulated FGF/FGFR function plays a role in pituitary tumorigenesis, and particularly in prolactinoma development.

CD31 and CD34

Different markers of MVD such as CD 31 and CD 34, Factor VIII (factor eight-related antigen), and ulex europaeus agglutinin I, have been used to evaluate angiogenesis. CD31 and CD34, both endothelial cell antigens, are sensitive markers of microvessels. They stain the majority of tumor vessels, both mature and new vessels. Even though antibodies to CD31 are not completely specific for endothelial cells, as they may also detect plasma cells, they are widely used for MVD appraisal, and results generally correlate with those obtained with CD34. Using these endothelial cell markers, some authors have found more prominent vasculature in prolactinomas, and others found that these tumors had the lowest while TSH secreting adenomas had the highest MVD. It has also been reported that ACTH secreting tumors had the lowest MVD, while other authors found that GH secreting adenomas had the lowest, or the highest MVD. Finally, some authors did not find any significant difference in MVD between the hormonal subtypes. These results point to the complexity of evaluation of vascularity in the adenomatous pituitary, and, as suggested by Itoh et al. (2003) angiogenesis may be revealed in the alteration of diameter or shape of the blood vessels.

With regard to the relation between MVD and sex or age of the patients, contradictory findings have also been reported. Jugenburg et al. (1995) reported no significant correlations, whereas Turner et al. (2000a) found tumor MVD clearly decreased with age in GH producing adenomas, and there was a trend in other tumor types from older patients to have lower MVD. In contrast, a positive correlation between age and MVD has also been reported. We described that in pituitary adenomas: CD31 expression was not different between sexes, and did not correlate with patients' age when all adenomas were considered. Nevertheless, if only non functioning adenomas were analyzed we found a positive correlation of CD31 with increasing age (Cristina et al. 2010), in agreement with other authors (Vidal et al. 2001), and therefore age may have an influence on the extent of neovascularization of non functioning adenomas.

Interestingly enough, we described a high correlation of VEGF and CD31 expression for all adenoma types, and for prolactinomas and nonfunctioning adenomas in particular (Cristina et al. 2010). This is in contrast to results published by other authors in which MVD did not correlate with VEGF expression. Therefore, the strong positive association of VEGF and CD31 expression found in pituitary adenomas suggests the participation of tumor vascularization in adenoma development.

On the other hand, proliferation markers (PCNA and Ki67) do not correlate with the angiogenic markers CD31 and VEGF, as described by us and others. This suggests that the rate of epithelial and tumor cell proliferation in pituitary tumors is not directly related to neovascularization, and other factors, such as primary genetic alterations or alteration of apoptotic pathways, may directly affect the rate, invasiveness and tumor behavior. In this respect, a positive relationship was observed between the expression of bcl-2, an antiapoptotic protein, and increasing MVD, suggesting an association between angiogenesis and cell survival.

Dopamine D2 Receptors and Angiogenesis

A relationship between the dopaminergic D2 receptor (D2R) and endothelial cell proliferation within tumors has been proposed. Dopamine and other related catecholamine neurotransmitters that interact with the D2R selectively inhibit VEGF–induced angiogenesis and inhibit the growth of malignant tumors as well as the vascular permeabilizing and angiogenic activities of VEGF. Besides, in two outbred lines of Wistar rats, which present high and low dopaminergic reactivity, respectively, VEGF expression was lower in the first group, and this group was more resistant to tumor implantation and developed significantly fewer lung metastases.

It is well established that D2R is the principal receptor involved in prolactin inhibition at the pituitary level, and in αMSH regulation at the intermediate pituitary lobe. Therefore, as expected, D2R knockout (*Drd2*$^{-/-}$) mice generated by targeted

mutagenesis have chronic hyperprolactinemia, pituitary hyperplasia, and a moderate increase in serum αMSH. We also showed that they are growth retarded evidence and alteration in the GH-IGF-I axis. After 16 months of age, highly vascularized adenomas develop, especially in females, but also in males. Prominent vascular channels as well as extravasated red blood cells are not contained in capillaries or peliosis, a common finding in the hyperplastic and adenomatous *Drd2*$^{-/-}$ pituitaries. As described, peliosis has been found in different tumors that secrete VEGF.

We found that VEGF mRNA and protein expression were increased in pituitaries from *Drd2*$^{-/-}$ female mice when compared to age-matched wild-type female mice (Cristina et al. 2005). Pituitary VEGF production is stimulated by estrogen in rat pituitaries and the somatolactotroph cell line GH3. Nevertheless, estrogen levels are not increased in *Drd2*$^{-/-}$ female mice, indicating that increased pituitary VEGF expression is mainly dependent on the lack of dopaminergic control. In experiments with wild-type female mice we found that prolonged treatment with the D2R antagonist, haloperidol, enhanced pituitary VEGF protein content and prolactin release (Cristina et al. 2005), and there was a significant correlation between pituitary VEGF levels and serum prolactin after haloperidol treatment. These results support the notion that dopamine acting at the D2R inhibits pituitary VEGF expression.

Interestingly, we found that the main source of VEGF in the hyperplastic pituitary were follicle stellate cells and not lactotrophs (Cristina et al. 2005). Follicle stellate cells represent 5–10 % of pituitary cells and are an important component of paracrine communication within the pituitary. They are detected by their content of the glial protein S100, they form follicles, are star shaped, and have long processes in between the secretory cells of the pituitary. They also contain FGF-2, follistatin, and interleukin 6. Because D2Rs have been described in lactotropes and not in follicle stellate cells it may be inferred that a paracrine-derived factor from lactotropes is acting on follicle stellate cells to increase VEGF expression. These data indicate that the D2R is linked to pituitary VEGF expression. In dopamine agonist resistant prolactinomas a decrease in number or function of D2Rs has been proposed (Caccavelli et al. 1994), and we have found highly expressed VEGF in a dopamine agonist giant prolactinoma (Mallea-Gil et al. 2009).

VEGF and its receptor may become supplemental therapeutic tools in dopamine-resistant prolactinomas. In this regard, in recent years, antiangiogenesis has been publicized as a novel alternative or supplement to conventional cancer therapy, and a variety of regimens that prevent tumor angiogenesis and/or that attack tumor blood vessels have met with remarkable success in treating mouse cancers.

Overexpression of VEGF by tumour cells can be targeted by:

- Antibodies against VEGF (Bevacizumab).
- Antibodies against VEGF receptors.
- Soluble VEGF receptors (VEGF-TRAP) that bind circulating VEGF.
- Catalytic RNA molecules (ribozymes), which cleave VEGF receptor mRNA.
- Orally available molecules that selectively block or prevent activation of VEGF receptor tyrosine kinases.

Despite the spectacular successes reported in the treatment of mouse tumors, the first clinical trials were discouragingly negative. This could be related to the fact that most of the patients treated in the beginning had advanced disease and had already failed conventional treatments. Also, antiangiogenesis therapy differs fundamentally from chemotherapy, and optimal implementation was needed.

Several agents targeting the VEGF ligand are now being developed in different clinical trials around the world to treat colon, rectal, breast, lung and other cancers. Bevacizumab (Avastin™), an anti-VEGF monoclonal antibody that inhibits formation of neovasculature and tumor growth in many human cancer cell lines, has a proven survival benefit in metastatic colon rectal cancer, and has now been approved by the FDA in combination with intravenous 5-FU-based chemotherapy as a treatment for patients with first-line metastatic cancer of the colon or rectum (Hurwitz et al. 2004).

Conclusions

In pituitary adenomas an altered expression of growth factors and their receptors has been observed (Asa and Ezzat 2002; Ezzat 2001; Melmed 2003; Renner et al. 1996; Turner et al. 2003). Although it is unlikely that these alterations play a causative role in pituitary tumor pathogenesis, intratumoral changes of these factors at their receptors may result in a permissive microenvoronment that contributes to excessive hormone production and loss of growth control in pituitary adenomas. Each pituitary tumor of clonal origin represents the multifactorial result of failure of different regulatory events. In this regard, pro- and anti-angiogenic growth factors such as FGF-2, VEGF, and others, may determine the final angiogenic phenotype of pituitary tumors, and thus subsequent tumor behavior. Furthermore, we believe that the study of angiogenic factor expression in aggressive prolactinomas with resistance to dopamine agonists will yield important data in the search of therapeutical alternatives.

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