Pituitary 8: 17–23, 2005

DOI: 10.1007/s11102-005-5081-6

Angiogenesis in Prolactinomas: Regulation and Relationship with Tumour Behaviour

N. Garcia de la Torre [1], *H.E. Turner* [2], *and J.A.H. Wass*[2]

[1] *Servicio de Endocrinología, Hospital Clínico San Carlos, Profesor Martín Lagos s/n, 28040, Madrid, Spain;* [2] *Department of Endocrinology, The Oxford Centre for Diabetes, Endocrinology and Metabolism, Churchill Hospital, Headington, Oxford OX3 7LJ, UK*

Abstract. **Tumours are dependent on angiogenesis for growth and inhibition of angiogenesis has become a target for antineoplastic therapy. In the pituitary, unlike other tissues, vascularization is lower in adenomas compared to the normal gland. Despite this finding, a relationship between increased vascularity and several aspects of prolactinoma behaviour such as size, invasiveness, surgical outcome and malignancy, has been demonstrated. The process of angiogenesis is the result of a balance of stimulating and inhibiting factors. It is likely that an interaction between gene expression (such as pituitary tumour transforming gene (PTTG) and a novel gene located within the *Edpm5* quantitative trait locus), hormonal stimuli including oestrogens, dopamine, 16 kDa fragments of prolactin and proangiogenic and antiangiogenic growth factors (for example, vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF-2), determine the final angiogenic phenotype of prolactinomas, and thus subsequent tumour behaviour. The elucidation of all the factors involved in the regulation of angiogenesis and their interactions might open new possibilities in the treatment of prolactinomas, especially in those cases with resistance or intolerance to dopamine agonists.**

Key Words. **angiogenesis, prolactinoma**

Introduction

Angiogenesis describes the process of development of new blood vessels from existing vasculature. Physiological angiogenesis during adult life is mainly restricted to the female reproductive cycle and wound healing. With the exception of these two processes, angiogenesis is usually inhibited in the normal tissues of the adult [1], but may be activated in some pathological diseases, for example psoriasis [2], retinal neovascularization [3], arthritis [4], and malignancy [5]. Angiogenesis plays a crucial role in tumour growth, in that it promotes oxygenation, nutrient perfusion, and the removal of metabolic waste [6]. There are now several experimental [7] and clinical data [8] showing that growth of solid tumours is angiogenesis-dependent [9]. In addition, angiogenesis (measured as tumour microvessel density) has been shown to be related to tumour behaviour. In many human tumours including prostate, breast, stomach, and bladder, increased angiogenesis has been shown to be correlated with development of metastases [10], poor prognosis [11], and reduced survival [12,13]. Angiogenesis is a complex multistep process, involving stimulation by various proangiogenic growth factors, and reduction in inhibitors of angiogenesis. It is the net balance of the proangiogenic factors and the inhibitors of angiogenesis that determine the final angiogenic phenotype of the tumour [14].

The prediction of pituitary tumour behaviour and the mechanism of differences in pituitary tumour growth and aggressiveness are still unclear. The determinants of tumour growth or limitation of size, tumour invasion of bone and other surrounding structures, and hormonal activity remain to be elucidated. This review discusses the recent work investigating whether differences in angiogenesis may play a role in determining different aspects of prolactinoma behaviour and assesses the potential mechanisms involved.

Morphological Vascular Changes in Pituitary Adenomas

The observation of reduced vascularization in the parenchyma of pituitary tumours compared to autopsy specimens of normal pituitary tissue was first reported by Schechter [15]. Subsequent studies assessed vascularization in a more comprehensive manner using immunostaining for different endothelial markers [16–18] and confirmed that the microvascular density (MVD) of pituitary adenomas is significantly lower than in the normal gland.

The pattern of higher vascularization in normal pituitary tissue compared with tumourous specimens is in contrast to a wide variety of tissues, where an

Address correspondence to: J.A.H. Wass, Department of Endocrinology, The Oxford Centre for Diabetes, Endocrinology and Metabolism, Churchill Hospital, Headington, Oxford OX3 7LJ, UK. Tel.: +44 (0)1865 857310; Fax: +44 (0)1865 742348; E-mail: john.wass@noc.anglox.nhs.uk

increase in the proliferation requires an increase in the vascular supply [19,20]. Even pre-malignant lesions have also been shown to be more vascular than normal tissue. Pre-carcinoma of the cervix exhibits high levels of angiogenesis [21]. Histologically normal lobules from the breast harbouring cancer have been shown to be significantly more angiogenic than lobules from breast without cancer [22] and transgenic mice with an oncogene in the pancreatic β-cells demonstrates increased angiogenesis in hyperplastic islets prior to the development of frank neoplastic change [23]. Other benign endocrine tumours as parathyroid adenomas and parathyroid hyperplasia showed an increased MVD compared to normal glands [24]. However, the decreased vascularization in a tumour compared with normal tissue is a common finding in other endocrine tissues as thyroid (Garcia de la Torre *et al.*, manuscript in preparation) and adrenal cortex [25].

Low vascular density and/or inhibition of angiogenesis is unusual for a tumour but may play a role in the usually slow growth of pituitary adenomas and may at least in part explain the common finding of incidental non progressive pituitary microadenomas in 10% of normal glands [26]. Alternatively, the low growth rate of these tumours may not influence the metabolic demand significantly, so that vascularization does not limit the growth.

Vascular Supply in Pituitary Tumours

Unlike other sites of tumour formation, the anterior pituitary has a dual blood supply. The hypothalamo-pituitary portal supply is the main source, carrying blood from the median eminence with hypothalamic releasing and inhibitory factors, but there is an additional direct arterial supply from the loral and capsular arteries [27]. The liver also has a dual blood supply (portal and direct arterial blood supply), and most metastases derive their blood supply directly from the hepatic artery rather than the hepatic portal vein [28]. The source of the blood vessels supplying the pituitary adenomas is unclear, although there are several studies suggesting that a direct arterial supply from the systemic circulation may develop. These reports include radiological data from angiography [29] and dynamic magnetic resonance imaging [30]. Anatomical studies in human pituitary tumours have reported an arterial blood supply [31,32], that has been further confirmed by an animal model of oestrogen-induced lactotroph hyperplasia and tumorigenesis in rats [33, 34]. The apparently lower MVD of pituitary tumours may perhaps reflect a completely or partially *de novo* blood supply from the extraportal system. Therefore, although the tumours are less vascular overall, they may have induced new vessel development from the systemic circulation, escaping hypothalamic influences on hormone production and the lower MVD may be the end result of an increase in angiogenesis after all.

Relationship Between Angiogenesis and Prolactinoma Behaviour

Microprolactinomas have been shown to be significantly less vascular than macroprolactinomas [17]. This finding fits with the clinical observation that microprolactinomas rarely progress in size and are a distinct clinical entity from macroprolactinomas, which may grow to a considerable size, suggesting that they are not part of the same pathological process [35]. In addition to tumour size, other aspects of tumour behaviour such as invasiveness and malignancy are also related to MVD. Consistent with the view that the occurrence of metastases and invasion are dependent upon angiogenesis it has been shown that MVD is significantly higher in pituitary carcinomas of prolactin-producing type than in their corresponding pituitary adenomas [36] and invasive macroprolactinomas are significantly more vascular than non-invasive macroprolactinomas [37]; in fact, the vascular density of invasive macroprolactinomas reached in this study that of the normal pituitary gland. Furthermore, the pre-operative prolactin level from both microprolactinomas and macroprolactinomas was positively correlated with vascular density, and macroprolactinomas that were cured by trans-sphenoidal surgery were significantly less vascular than those not cured by surgery [37]. This is likely to be related to differences in tumour invasiveness and difficulty in completely resecting more vascular prolactinomas.

Turner and co-workers assessed the proliferative capacity of a cohort of pituitary adenomas using the Ki-67 labelling index (LI) and analysed its relationship with angiogenesis and tumour behaviour [38]. The Ki-67 LI of macroprolactinomas was significantly higher than all other tumours, including microprolactinomas which had the lowest Ki-67 LI. This is in keeping with the fact that macroprolactinomas often behave more aggressively than other benign pituitary tumours and often show local invasion. The significant difference between Ki-67 LI of macroprolactinomas and microprolactinomas is in support of other studies [35] that demonstrated that microadenomas producing prolactin had significantly lower Ki-67 LI than macroadenomas. However, this and other studies did not find an association between Ki-67 LI and MVD [36,38]. The lack of association between Ki-67 LI and MVD in pituitary tumours is not entirely surprising as the LI is simply a measure of the proportion of cells that have entered the cell cycle and gives no idea of the speed of progress through the cycle and therefore tumour growth, which in pituitary adenomas is usually slow. In theory, it might be expected that MVD could limit tumour growth by limiting cell proliferation although this is clearly not the case in the pituitary. It has been suggested that the lack of correlation may be because the MVD is more indicative of the surviving rather than the proliferating fraction of cells [38]. In the same study

bcl-2 expression (a protein that inhibits apoptosis and gives cells that over-express it a survival advantage) was significantly associated with MVD, with higher bcl-2 expression found in the more vascular tumours. The positive association between bcl-2 expression and MVD suggests that the control of angiogenesis and programmed cell death may be related. Perhaps a switch to an angiogenic phenotype and bcl-2 expression are both early events in pituitary tumorigenesis or that non-cycling but surviving cells support angiogenesis.

Very recently, the structural organization of the microvascular bed in prolactin-producing adenomas and carcinomas has been studied by assessing microvascular structural entropy (MSE as a measure of the degree of disorder in a system), and its relationship with MVD and tumour cell proliferation analysed [39]. The morphometric study demonstrated statistically significant differences in Ki-67 LI, MVD, and MSE between prolactin-producing adenomas and carcinomas. Unlike Ki-67 LI and MVD, the MSE values were significantly higher in adenomas than in carcinomas. These results indicate that prolactin-producing carcinomas have a less chaotic distribution of vessels than benign adenomas. In contrast to a lack of correlation between MVD and Ki-67 LI [36,38], a strong negative correlation was found between MSE and Ki-67 LI. It therefore appears that regular, less chaotic microvascular geometry contributes to increased proliferative activity in prolactin-secreting tumours.

The presence of matrix metalloproteinase-9 (MMP-9) and also the level of expression are related to tumour invasiveness in prolactinomas [40]. The MMP's are a family of zinc-containing endopeptidases that are able to degrade the extracellular matrix and allow angiogenesis and tumour invasion. In this study invasive macroprolactinomas were significantly more likely to express MMP-9 (10 out of 11) than noninvasive macroprolactinomas (1 out of 8). In addition invasive prolactinomas showed a higher density of MMP-9 staining than noninvasive prolactinomas. There was no difference between noninvasive prolactinomas and normal pituitary gland in terms of MMP-9 positivity. These data suggest that MMP-9 expression is related to invasiveness and aggressive tumour behaviour in prolactinomas, and its presence or absence may act as a possible marker of tumours that are more likely to invade. There was no relation between MMP-9 expression and the Ki-67 LI or bcl-2 expression, however, the MVD was significantly higher in MMP-9 positive tumours compared with MMP-9 negative tumours [40]. It is not known whether the increased angiogenesis acts as a stimulant to MMP-9 expression to allow further endothelial migration, or whether the stimulus to angiogenesis also leads to increased MMP-9 expression and, therefore, potentiates tumour invasion. The gene for MMP-9 has been localized to chromosome 20 [41], and targeted disruption of the MMP-9 gene in mice leads to reduced angiogenesis, suggesting that MMP-9 may play a role in controlling angiogenesis [42].

A relationship between cyclin expression and tumour size has been shown in pituitary adenomas [43]. The cyclins are proteins which play an important role in control of the cell cycle during cell proliferation, being essential for passage through specific stages of the cell cycle. It may be important as one mechanism of growth of pituitary adenomas, and is in keeping with the different Ki-67 LI and differences in angiogenesis shown in prolactinomas of different sizes. It suggests that there is perhaps a switch which enables tumours to enlarge in size with higher cyclin expression, increased Ki-67 LI and increased angiogenesis. This may explain the relatively common finding of pituitary incidentalomas at autopsy or on MRI, which are less than 1 cm in size and do not enlarge [26] and also the fact that microprolactinomas usually remain as microadenomas, rather than grow into macroprolactinomas.

Regulation of Angiogenesis in Prolactinomas

The sinusoid-capillary network of the anterior lobe of the pituitary gland has a fenestrated layer of endothelial cells, as in all endocrine organs, which allows soluble factors (growth factors or hormones) to diffuse into the surrounding tissue and vice versa. In that way proangiogenic and antiangiogenic growth factors of the pituitary can bind to endothelial cells, and hormones produced in peripheral endocrine glands (e.g. ovary) or their synthetic analogues can influence hormone and growth factor production by tumour cells. In addition to this interaction between hormones and growth factors, genetic events involved in pituitary tumour pathogenesis may play an important role in the regulation of angiogenesis in prolactinomas.

Regulation of angiogenesis by growth factors

Ferrara and co-workers were the first to study folliculostellate cells function following enzymatic dispersal of bovine anterior pituitary gland, and *in vitro* culture. They demonstrated the production of fibroblast growth factor-2 (FGF-2) [44], and a then new growth factor — vascular endothelial growth factor (VEGF) — [45], from these cultures. VEGF plays a key role in both physiological and pathological angiogenesis through the increase of proliferation and migration of endothelial cells [46] and also increasing endothelial permeability by inducing fenestrations in the endothelium [40]. In addition, VEGF functions as an anti-apoptotic factor promoting the survival of endothelial cells in newly formed vessels [47]. Its presence has been demonstrated in the pituitary gland of several species and in the GH3 cell line [48,49] and approximately 90% of human pituitary tumours showed measurable VEGF secretion *in vitro* [50]. VEGF expression could also be detected by immunohistochemistry and *in situ* hybridization in pituitary adenomas [51,52].

FGF-2 is a potent angiogenic factor produced by endothelial, stromal and tumoural cells as well as released from the extracellular matrix, and stimulates proliferation of endothelial cells [53]. Although elevated FGF-2, in addition to VEGF, plasma concentrations have been demonstrated in patients harbouring pituitary tumours [54], there were no data directly relating this factor to angiogenesis in pituitary adenomas until the recent discovery of the human pituitary tumour transforming gene (PTTG) and its involvement in pituitary tumour pathogenesis (see below).

Recently, Basu and colleagues [55] have provided evidence that dopamine (DA) can selectively inhibit VEGF-induced angiogenesis of mouse ovarian tumour *in vivo*, as well as VEGF-induced endothelial cell proliferation and migration of cultured human umbilical vein endothelial cells (HUVEC) *in vitro*. Moreover, DA can inhibit VEGF receptor type 2 phosphorylation in HUVEC. This effect of DA is likely to be mediated through DA_2-receptors detected on endothelial cells, because other DA_2 agonists like bromocriptine and quinagolide had the same effect, and DA_1, DA_3, and DA_4 antagonists could not reverse the effect of DA. The inhibitory effect of DA was selective to VEGF and did not affect the effect of other factors as FGF-2. These findings might provide a novel approach to angiogenic therapy. However, several studies could not show a statistically significant difference in MVD between bromocriptine-treated and untreated prolactinomas [36,37].

Some angioinhibitors have been shown to have inhibitory effects in prolactin-secreting pituitary adenomas induced by prolonged treatment with oestrogens in Fischer rats 344 [56]. Fugmagillin and its analogue TNP-470 are known to inhibit endothelial cell proliferation selectively. Both angiogenesis inhibitors attenuated the stimulatory effect of diethyl-stilboestrol on prolactin production and diminished prolactin cell density and inhibited cell proliferation. As expected both angioinhibitors suppressed neo-vascularization within the anterior pituitary, thus these anti-tumour and anti-prolactin effects might be mediated indirectly through the inhibition of angiogenesis.

Hormonal regulation of angiogenesis

There is increasing evidence that hormones play an important role in the control of endothelial cell function and growth. Oestrogens have been shown to increase expression of VEGF. Protein expression of VEGF as well as its receptor type 2 increased during the development of oestrogen-induced prolactinomas in the pituitary of rats and this was associated with the growth and enlargement of blood vessels [57]. These findings suggest a role in the modulation of pituitary tumour angiogenesis that could be a part of the mechanism by which oestrogens cause pituitary hyperplasia and possibly prolactinoma formation. Further studies showed a strong inhibition of oestrogen-induced lactotroph tumour angiogenesis by methoxyestradiol; furthermore, VEGF expression was downregulated, concomitant with suppression of tumour angiogenesis [58].

Many endogenous inhibitors of angiogenesis have been shown to be cleaved products of other larger proteins. The 16 kDa N-terminal fragment of human prolactin (16K PRL) is a potent antiangiogenic factor *in vivo* in the chicken chorioallantoic membrane (CAM) assay [59]. 16K PRL inhibits FGF-2 and VEGF-induced cell proliferation of cultured bovine and human capillary endothelial cells [60]. Further studies have shown that 16K PRL inhibits FGF-2 induced angiogenesis *in vivo* in the rat cornea model [61]. Although 16K PRL has been demonstrated in the pituitary gland of the rat [62], there are no data on its potential role in the human pituitary gland and pituitary tumours.

Genes and angiogenesis

The recently isolated pituitary tumour-derived transforming gene (PTTG) has been shown to be highly expressed in malignant human cell lines and pituitary tumours [63]. Pituitary PTTG is regulated *in vivo* and *in vitro* by oestrogens. In the rat prolactinoma model, oestradiol was shown to induce PTTG expression early in pituitary transformation (normal cell to hypertrophic/hyperplastic cell), followed within 24 hours by increased FGF-2 and VEGF expression associated with pituitary angiogenesis [64]. Using the same animal model, selective anti-oestrogen treatment blocked oestrogen-induced pituitary PTTG expression and inhibited lactotroph tumour growth [65]. PTTG induces an angiogenic phenotype in both *in vitro* and *in vivo* angiogenesis models, and increased PTTG mRNA is associated with angiogenic phenotype in human tumours [66]. It has been suggested that FGF-2 may be the effector for PTTG driven angiogenesis, since rat pituitary tumours with higher vascularity showed increased PTTG expression, and anti-FGF-2 antibodies inhibited PTTG stimulation of new blood vessel formation *in vitro* [67]. Further studies reported that PTTG also stimulates VEGF expression *in vitro* independent from FGF-2 up-regulation and that VEGF and PTTG mRNA expression were highly correlated in human pituitary tumours [68]. Thus, PTTG overexpression seems to be an important mechanism of pituitary tumorigenesis and angiogenesis.

Very recently *Edpm5* has been found to specifically regulate the switch to the angiogenic phenotype, independent of neoplasia, in oestrogen induced prolactinoma in rats [69]. *Edpm5* is one member of a group of quantitative trait loci which are responsible for the difference in susceptibility to oestrogen induced prolactinoma between the Fischer 344 (F344) and Brown Norway (BN) strains. Upon chronic oestrogen treatment F344 rats develop large, hemorrhagic and invasive pituitary prolactinomas, which exhibit both tumour angiogenesis and neoplasia. In contrast, BN do not develop a tumour despite an oestrogen-induced increase in lactotroph density. The segment of rat chromosome bearing *Edpm5* from BN was introgressed into

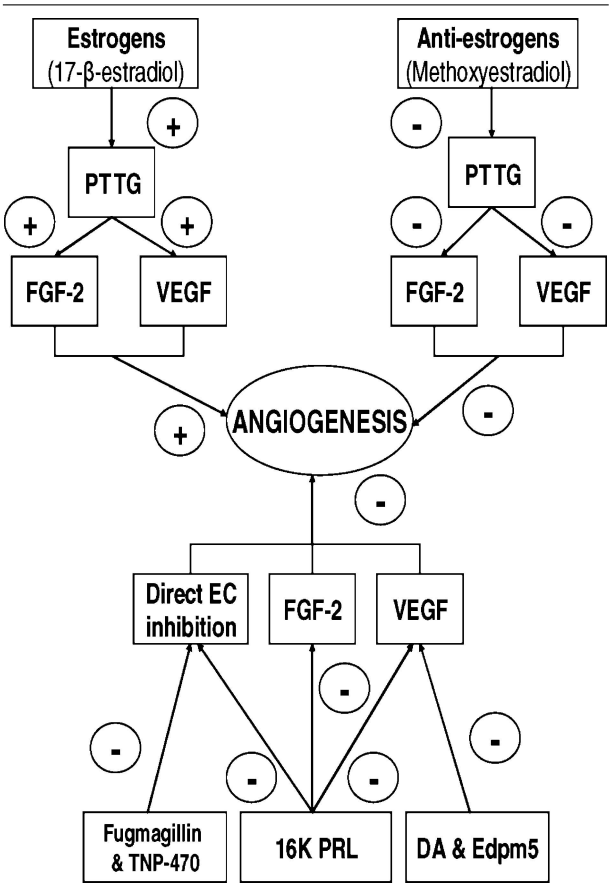

Fig. 1. *Proposed model of hormonal regulation of angiogenesis in prolactinomas. PTTG: Pituitary tumour transforming gene, FGF-2: Fibroblast growth factor-2, VEGF: Vascular endothelial growth factor, EC: Endothelial cells, 16K PRL: 16 kDa fragment of prolactin, DA: Dopamine.*

the F344 strain background, therefore phenotypic differences between F344 and congenic rats must be due to a gene(s) within the chromosomal interval encompassing *Edpm5*. Tumour growth in congenic rats was indistinguishable from that of the F344 strain. However, prolactinomas in congenic rats had a non-angiogenic phenotype. After chronic oestrogen treatment, there was no increase in microvessel count over untreated controls in congenic rats, whereas F344 rat tumours showed a significant increase in vascularization. The congenic strain also failed to express VEGF at the high levels seen in the F344 rat pituitary after oestrogen treatment. Therefore, one gene that has a large impact preventing the switch to angiogenic phenotype must reside within the chromosomal interval that is the *Edpm5* quantitative trait locus.

Conclusions

Proangiogenic and antiangiogenic growth factors, hormonal stimuli and genes constitute an integrated regulatory system that determines the angiogenic phenotype of prolactinomas and possibly some aspects of tumour behaviour (Fig. 1). However, the regulation of angiogenesis in prolactinomas is not fully elucidated and the potential efficacy of inhibition of this process is unclear. Nevertheless, the regulation of angiogenesis by drugs and some recently found genes which regulate the angiogenic switch might open new possibilities in the treatment of prolactinomas, specially in those cases with resistance or intolerance to dopamine agonists.

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