EXPERIMENTAL STUDY

Correlation of VEGF production with IL1 α and IL6 secretion by human pituitary adenoma cells

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Abstract

Objectives: Vascular endothelial growth factor (VEGF) is considered to be the most important angiogenic factor involved in the neovascularisation of solid tumours. Regulatory molecules include cytokines and growth factors. Interleukin (IL)1 and IL6 have both been shown to regulate VEGF levels in a variety of tissues. The role of cytokines in the pathogenesis of pituitary tumours remains unclear. We have examined the expression of VEGF and its relationships with IL1 and IL6 in the human pituitary tumour cell line HP75 and a series of human pituitary tumours. We have also looked at the relationship of tumour volume and invasive status to VEGF secretion.

Methods: Surgically resected tumours were routinely cultured in single-cell suspension at 200 K/well (standard unit for culture of dispersed primary pituitary adenoma cells). We measured VEGF, IL1 α and IL6 levels by ELISA. Tumour volume and invasion grade were assessed by preoperative magnetic resonance imaging.

Results: VEGF was detected in conditioned medium of HP75 cells (900 ± 52 pg/ml) and in 82% of tumours tested (range 26–16 464 pg/ml). Tumour volume and secretion of VEGF were significantly associated with levels of IL6 (volume, $P = 0.056$; VEGF, $P < 0.001$ (P values based on Spearman's test)) and IL1 α produced (volume, $P < 0.005$; VEGF, $P < 0.001$). Invasive tumours showed a higher basal secretion of VEGF than that of the non-invasive type; however, this difference was not significant. Addition of exogenous IL1 α , but not IL6, significantly increased VEGF production.

Conclusions: The significant associations between VEGF and the levels of IL6 and IL1 α suggest an important role for these cytokines in the development of these tumours.

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Introduction

Vascular endothelial growth factor (VEGF) is known to be a potent mitogen for vascular endothelial cells and an inducer of physiological and pathological angiogenesis (1). Angiogenesis, the growth of new capillaries from pre-existing blood vessels, is essential for tumours to grow beyond a few millimetres in diameter (2, 3). VEGF is also essential for normal developmental vasculogenesis and angiogenesis, as both null (–/–) and heterozygote (+/–) animals are embryonic lethals (4, 5).

IL6 is known to be expressed in the normal pituitary gland by folliculostellate (FS) cells, but not secretory cells and all subtypes of human pituitary adenomas. First demonstrated by Jones *et al.*, secretion of IL6 by these tumours was seen to be independent of subtype (6), as confirmed by IL6 mRNA detection (7, 8). The production in the normal gland is believed to be by FS cells (9); however, in pituitary adenomas, it is the tumour cells which are the source of production (10). The role of other cytokines released by pituitary adenomas has received less attention. Expression of IL1 α and

β has been demonstrated only by RT-PCR, with 3 of 17 tumours positive for IL1 α and 6 of 17 positive for the β isoform (8).

The role of cytokines in the pathogenesis of human pituitary tumours has proven difficult to elucidate, as primary cultures from most pituitary tumours do not display significant growth to allow extensive study. Therefore, in addition to our use of primary tissue, we have used the recently developed HP75 cell line, the only established human pituitary tumour cell line (11). This cell line, derived from a clinically non-functioning plurihormonal adenoma, was transfected with the SV40 virus, and it retains a number of differentiated functions, including chromogranin A expression and positive staining for luteinising hormone (LH).

In normal tissues, vascular quiescence is maintained by the dominant influence of endogenous angiogenesis inhibitors over angiogenic stimuli, whereas tumour angiogenesis is induced by increased secretion of angiogenic factors and/or by downregulation of angiogenesis inhibitors (12). VEGF is regarded as the major angiogenic factor during epithelial carcinogenesis in a large

number of human cancers and metastases (13, 14). It is ubiquitously expressed in many human tumours, including those of the lung (15), breast (16) and ovary (17), and in glioblastoma multiforme (18). The key regulator of VEGF expression is hypoxia (19, 20); however, a range of cytokines and growth factors have been shown to regulate and correlate with VEGF expression. Interleukin (IL)1 α / β stimulates production of VEGF in a diverse range of cell types, including human cultured synovial fibroblasts (21), peripheral blood mononuclear cells (22), vascular smooth muscle cells (23) and cardiac myocytes (24), and also in rat ovary cells (25). In parallel with this, another cytokine seen to regulate VEGF in a variety of tissues is IL6. It has been suggested that IL6 may induce angiogenesis not only through its effects on VEGF expression but also by its direct stimulation of the motility of cells such as endothelial cells (26, 27). In rat skeletal muscle myoblasts and glioma cells, Cohen *et al.* found that IL6 increases VEGF mRNA expression by levels comparable to those seen with hypoxia (28). Addition of exogenous IL6 to a cervical cancer cell line *in vitro* has also been found to show a time- and dose-dependent increase of VEGF expression (29). While Salgado *et al.* found that exogenous IL6 showed no effect on VEGF levels, removing basally secreted IL6 by a neutralising antibody significantly lowered the VEGF secretion by a human megakaryoblastic cell line (30). A number of studies measuring the basal levels of VEGF and IL6 have been conducted; a positive correlation has been observed between serum levels of IL6 and VEGF in patients with advanced metastatic cancer (31). More recently, serum levels of IL6 and VEGF have shown a positive correlation in patients with breast and gastric carcinomas (32–34).

The presence of VEGF has been widely reported in human pituitary adenomas and various pituitary cell lines (35–38). However, the factors regulating its expression in these tumours have not been widely investigated. In the pituitary FS cells, IL6 and glucocorticoids, which are regulators of IL6 secretion, were observed to increase and decrease VEGF secretion respectively (39). A more comprehensive study of the presence and regulation of VEGF secretion by Lohrer *et al.* looked at primary culture of human pituitary adenomas and four rat and murine pituitary cell lines (35). They detected VEGF secretion by the majority of the pituitary adenomas and all the cell lines. VEGF levels were enhanced in some of the primary tumours by tumour growth factor (TGF) α , pituitary adenylate cyclase activating polypeptide (PACAP) and 17 β -oestradiol, and inhibited by the glucocorticoid, dexamethasone. The pituitary adenoma cell lines were responsive only to dexamethasone, and this agent inhibited VEGF secretion. The TtT/GF FS line showed the greatest responsiveness to the stimuli, and basal values were augmented by a wide range of factors, including IL6, PACAP, TGF α , insulin-like growth factor (IGF)-I and the somatostatin analogue, octreotide (35).

The generation of a direct arterial supply during neovascularisation has important consequences for pituitary tumour cells, as it not only allows tumour expansion but also uncouples the adenoma cells from the portal blood system and thus from hypothalamic control (40, 41). VEGF is considered to be the most important angiogenic factor involved in the neovascularisation of solid tumours. Understanding the regulation of VEGF secretion will provide an important insight into the development of these tumours. We have investigated the expression of VEGF and its regulation in a series of 59 human pituitary adenomas and in the HP75 cell line. HP75, an available human pituitary tumour cell line, retains some differentiated functions and is known to secrete IL6 (42). Focusing our attention on the cytokines IL1 α and IL6 and their relationship to VEGF expression, we have also examined the relationship of VEGF with tumour invasive status and volume.

Materials and methods

Cell culture

HP75 cells were routinely cultured in DMEM (BioWhittaker/Cambrex, East Rutherford, NJ, USA) containing 15% horse serum (TCS Cellworks, Buckingham, UK) and 2.5% foetal calf serum (FCS) (Gibco BRL, Invitrogen, Paisley, UK). For growth and stimulatory studies, HP75 cells were plated in 24-well plates at 30 K/well in DMEM containing 2% foetal calf serum. After 24 h, the medium was changed, and test substances were added where required. After 72 h, conditioned medium was removed and stored at -80°C . All experiments were conducted in triplicate wells and repeated at least four times.

Human pituitary adenomas removed in routine transphenoidal surgery were prepared and dispersed for primary culture. The tissue was finely minced with a scalpel to maximise the tissue surface area for enzymatic digestion and incubated with dispase (Roche) (2.4 U/ml) in a shaking water bath at 37°C for from 30 min to 3 h, depending on the level of mucus present. Tissue dispersal was completed mechanically with needles of increasing gauge (19–25 g). Cells were spun at 1500 r.p.m. for 8 min, and the supernatant was poured off. Cells were resuspended, and hypotonic lysis was performed as described by Atkin *et al.* (43). Cells were counted with a haemocytometer and plated at 200 K/well into 24-well plates in 1 ml medium composed of Med199 (BioWhittaker) with 10% FCS, Fungizone (1.25 $\mu\text{g/ml}$) (BioWhittaker) and gentamicin (Hoechst, Roussel Laboratories, Cambridge, UK) (50 $\mu\text{g/ml}$). After 96 h, the medium was removed and stored, and it was replaced with Med199 containing the above ingredients but with only 2% FCS. Next, the test substances were added, and the cells were incubated for a further 72 h,

after which the conditioned medium was removed and frozen.

Alamar Blue

Cell growth was measured with Alamar Blue (Serotec, Oxford, UK). Alamar Blue was added to equal 10% of culture volume and incubated for 5.5 h at 37 °C; absorbance was then measured at 570 and 600 nm. All values are expressed as optical density (OD).

ELISA

IL6 was measured from cell-culture supernatants by ELISA with the IL6 Eli-pair antibody kit (IDS Ltd, Tyne and Wear, UK). Briefly, 96 well plates were coated with capture antibody and allowed to incubate overnight at 4 °C. Following washing and blocking of non-specific binding sites samples and standards (appropriately diluted) were added and then co-incubated with biotinylated anti-IL6 antibody for 1 h at room temperature. After further washing, the plates were incubated with horseradish peroxidase streptavidin (HRP-Strep) followed by 3,3',5,5'-tetramethylbenzidine (TMB) for 8–10 min. The reaction was stopped on addition of 1 M H₂SO₄, and absorbance was read at 450 and 630 nm. The sensitivity range was 6.25–200 pg/ml. The interassay variation was $\pm 5.4\%$ and the intra-assay variation was $\pm 2.8\%$.

VEGF was measured by a commercially available ELISA kit (AMS Biotechnology, Abingdon, UK), according to the manufacturer's instructions. The detection range was 20–2500 pg/ml. The intra-assay variation was $\pm 8.9\%$; that of the interassay was $\pm 11.1\%$.

IL1 α was measured by a commercially available ELISA kit (IDS), according to the manufacturer's instructions. The detection range was 10–1000 pg/ml. The intra-assay variation was $\pm 3.9\%$, and the interassay variation was 7.3%.

Radiology

The preoperative magnetic resonance imaging (MRI) scans of patients were reviewed by a neuroradiologist to determine their invasive status and volume. As we assumed the tumour to be spherical or egg-shaped, its volume was calculated by the formula for determining spherical volume, $v = 4/3\pi[(r_1 + r_2 + r_3)/3]^3$, where v is tumour volume in mm³, and r_1 , r_2 and r_3 are tumour height, and transverse and anteroposterior diameters respectively in mm. Invasive status was classified by the modified method of Hardy (44), placing adenomas in one of four grades, grades 1 and 2 showing no invasion, and grades 3 and 4 showing invasive activity.

Statistical analysis

All data groups were tested for normality by the one-sample Kolmogorov–Smirnov test, with subsequent analysis by either parametric or non-parametric tests as appropriate. Correlations between two variables showing non-normal distribution were assessed by the non-parametric Spearman correlation test. Comparison between two groups showing a normal distribution was made by Student's *t*-test and for non-parametric data by the Mann–Whitney U test.

Results

VEGF

VEGF levels were measured in the conditioned medium from a total of 59 tumours. Of these, 32 were null-cell adenomas, seven were growth hormone (GH)-secreting tumours, seven were adrenocorticotrophin hormone (ACTH)-secreting tumours (two silent), nine were silent gonadotrophinomas, one was a prolactinoma and three were plurihormonal tumours (GH/prolactin (PRL), PRL/GH/thyroid-stimulating hormone (TSH) and PRL/GH/TSH/follicle-stimulating hormone (FSH)). Of the 59 tumours examined in this series, 48 (82%) secreted measurable levels of VEGF into the culture medium (Fig. 1). The range of measurable basal secretions was 26–16 464 pg/ml. Of these, 29 of 32 null-cell and all the ACTH-producing tumours had measurable levels of VEGF. Seven of the nine gonadotrophinomas and five of the seven GH secretors also produced VEGF. The one prolactinoma was weakly positive, but neither the bihormonal nor the plurihormonal tumours produced measurable quantities of the protein.

Figure 1 Graph showing the secretion of VEGF (pg/ml) from cultured pituitary tumour per 200 K cells.

We saw constitutive expression of VEGF from HP75 cells with an average secretion of 900 ± 52 pg/ml (mean $\pm$ S.E.M.; range: 251–1668 pg/ml).

We have reported elsewhere the expression profile of IL1 α and IL6 by the HP75 cell line and primary cultures of human pituitary tumours; therefore, this is not discussed here.

IL6

The basal levels of IL6 secreted *in vitro* from the 59 tumours above were assessed. Of the 59 tumours, 58 expressed measurable levels of IL6, ranging from 7 to 1 368 070 pg/ml. The average basal level of IL6 protein secreted by HP75 cells was measured by ELISA and found to be 6167 ± 56 pg/ml (mean $\pm$ S.E.M.; range: 1596–25 840 pg/ml) over 72 h. It is unclear why there was a high degree of variability in the range of the basal secretions of VEGF and IL6 from HP75 cells.

There was a strong and positive correlation between the basal secretions of IL6 and VEGF by the human pituitary tumours cultured ($r^2 = 0.913$; $P < 0.0001$; $n = 46$) (Fig. 2). We also observed a positive association between tumour volume and level of IL6 secreted *in vitro* at a level very close to significance ($r = 0.388$; $n = 25$; $P = 0.056$).

IL1 α

The average level of IL1 α secretion from HP75 cells was 51 ± 6 pg/ml over 72 h per 30K cells. Fifty-two of the 59 tumours were assayed for IL1 α , of which 25 (47%) secreted detectable levels into the culture medium, ranging from 8 to 572 pg/ml.

Figure 2 Scatter plot showing a positive correlation between basal IL6 and VEGF secretions by human pituitary adenoma cells in culture ($r^2 = 0.913$, $P < 0.0001$; $n = 46$).

We saw that tumours that secreted measurable levels of IL1 α secreted significantly greater quantities of VEGF than those that did not (1363 ± 697 pg/ml, $n = 24$, vs 172 ± 52 pg/ml, $n = 27$; $P < 0.001$). These tumours also had significantly larger volumes than non-IL1 α secretors (IL1 secretors, 270.06 ± 61.74 ml, $n = 11$; IL1 non-secretors, 48.04 ± 18.25 ml, $n = 10$; $P < 0.005$).

Effect of IL1 α on VEGF secretion

HP75 cells and cultured primary tumours were treated with exogenous IL1 α , and VEGF release was assessed. Addition of 6.25 pg/ml IL1 α resulted in no significant change in the level of VEGF secreted by HP75 cells (basal 1055 ± 60 pg/ml; IL1 α 6.25 pg/ml: 858 ± 19 pg/ml; $P > 0.05$) (Fig. 2). On addition of higher doses of IL1 α , that is, 62.5, 625 and 6250 pg/ml, we saw significant increases of 143–149% in VEGF secretions compared with basal (basal 1055 ± 60 pg/ml; 62.5 pg/ml: 1513 ± 144 pg/ml, $P = 0.002$; 625 pg/ml: 1560 ± 124 pg/ml, $P = 0.0002$; 6250 pg/ml: 1574 ± 111 pg/ml; $P = 0.0001$) (Fig. 3).

Six primary cultures of pituitary tumours were treated with exogenous IL1 α at doses of 62.5, 625 and 6250 pg/ml, and their VEGF secretions were measured. One tumour produced no measurable levels of VEGF. Two tumours produced significantly increased levels of VEGF (139% and 173%) on stimulation by IL1 α at 6250 pg/ml (Fig. 4A, basal 448 ± 15 pg/ml; 6250 pg/ml: 623 ± 46 pg/ml, $P = 0.02$; Fig. 4B, basal 565 ± 22 pg/ml; 6250 pg/ml: 975 ± 146 pg/ml, $P = 0.045$). In another two tumours, we saw an increase in VEGF secretion with doses of IL1 α at 625 and 6250 pg/ml, but statistical significance (*) was achieved in both tumours only at the 625 pg/ml dose (Fig. 4C, basal 391 ± 92 pg/ml; *625 pg/ml: 660 ± 67 pg/ml (169%), 6250 pg/ml: 975 ± 146 pg/ml; Fig. 4D, basal 90 ± 5 pg/ml; *625 pg/ml: 122 ± 7 pg/ml).

Figure 3 Stimulatory effect of IL1 α on VEGF secretion in HP75 cells (mean $\pm$ S.E.M.; * $P < 0.05$ compared to basal).

Figure 4 Effect of exogenous IL1 α on VEGF secretion in the following tumour types: (A) silent FSH/LH, (B) ACTH, (C) silent FSH/LH, (D) null cell and (E) GH (mean $\pm$ S.E.M.; * $P < 0.05$).

(136%), 6250 pg/ml: 119 ± 13 pg/ml, $*P < 0.05$). One tumour was treated with a single dose of IL1 α at 625 pg/ml only, and while we saw an increase in VEGF secretion, this was not significant (Fig. 4E, basal 260 ± 43 pg/ml; 625 pg/ml: 358 ± 37 pg/ml, $P = 0.15$).

Effect of IL6 on VEGF secretion

We investigated the effect of the addition of exogenous IL6 on the VEGF secretion from HP75 cells and cultured human pituitary tumour cells. Addition of IL6 at all doses used showed no effect on the level of VEGF secreted by HP75 cells (basal 940 ± 80 pg/ml; IL6 1 ng/ml: 762 ± 129 pg/ml, IL6 10 ng/ml: 1078 ± 85 pg/ml, IL6 100 ng/ml: 1006 ± 93 pg/ml, $P > 0.05$).

The effect of the addition of IL6 to primary cultures with five tumours was assessed. One ACTH-secreting tumour had no measurable levels of VEGF. Each of the other four tumours (one null, one GH and two silent FSH/LH) investigated showed no significant change in the levels of VEGF secreted in response to addition of IL6 ($P > 0.05$).

Effect of IL1 α and IL6 neutralising antibodies on VEGF secretion

HP75 cells were treated with either an IL6 or an IL1 α neutralising antibody (Ab), and VEGF release was measured after 72 h of culture. No significant changes in VEGF production by these cells with either antibody were detected. The quantity of neutralising antibody used was calculated for the maximum dose to neutralise the average basal secretion of its cytokine by HP75 cells (IL6 Ab basal 1725 ± 59 pg/ml; 300 μ g/ml: 1721 ± 27 pg/ml, $P = 0.95$; IL1 α Ab basal 371 ± 24 pg/ml; 1000 ng/ml: 375 ± 54 pg/ml, $P = 0.96$).

VEGF, tumour volume and invasive status

We investigated whether or not the levels of secreted VEGF were associated with tumour invasive grade and/or volume. The invasive tumours (grades 3 and 4) had a higher average basal secretion of VEGF than those of the non-invasive type (invasive 2054 ± 1608 pg/ml, $n = 12$; non-invasive 608 ± 143 pg/ml, $n = 18$; $P = 0.867$); however, this difference was not significant. We did not observe any significant association between tumour volume and VEGF secretion ($r = -0.104$, $n = 22$, $P = 0.46$).

Discussion

The majority of human pituitary adenomas, independently of subtype, and HP75 cells synthesise and secrete VEGF *in vitro*. There is a strong correlation

between both the amount of IL1 α and IL6 secreted and the VEGF produced by cultured adenoma cells.

In the normal pituitary gland, VEGF is synthesised and released only by FS cells (45, 46). Mouse and rat pituitary tumour cell lines (AtT/20, GH₃ and AT3-1) and, it now seems, HP75 constitutively synthesise and release VEGF. FS cells are rarely found in human pituitary adenomas; the finding that the majority of these tumours produce VEGF suggests that the adenoma cells are the source. This further implies that tumour transformation results in the acquisition of the ability to produce this peptide.

VEGF has previously been shown to be present immunocytochemically in human pituitary adenomas. Lloyd *et al.* showed varying degrees of positivity with 93% of tumours from a large study ($n = 148$) (37). Lohrer *et al.* showed similar levels of VEGF expression with 93% of tumours positive (35). The slightly lower detection level in our study may be accounted for by the fact that our minimum detection limit for the VEGF assay was higher than that used by Lohrer *et al.* (20 vs 3 pg/ml).

Tumour size is dependent on adequate vascularisation. In pituitary adenomas, we and others have not demonstrated a correlation between VEGF production and tumour volume (35). However, there is a tendency for invasive tumours to have higher production than non-invasive ones, although this did not approach statistical significance. The reasons for this may be that other factors, such as tissue architecture, are important.

Although there is a positive correlation between IL6 and VEGF produced by adenomas, exogenous IL6 had no acute effect on VEGF release. This was confirmed by the lack of effect of IL6 neutralising antibody on VEGF secretion. This, however, does not exclude a chronic effect of IL6 on VEGF production and an effect which is dependent on the topographic relationship of cells in the undispersed tumour or the presence of hypoxia. This positive correlation of IL6 with VEGF has also been demonstrated in other tumours, such as breast and gastric malignancies (31, 32, 34).

On the other hand, IL1 α , which, as we have demonstrated here, has a positive correlation with VEGF, stimulated VEGF release by adenomas and HP75 cells in culture. However, the addition of neutralising antibody did not affect basal VEGF release.

The associations of IL6 and IL1 α with tumour volume suggest that the presence of these cytokines confers a growth advantage on the tumour, allowing its increased expansion. While we found no direct relationship between VEGF and tumour volume, it is more likely there is an indirect link between the two. VEGF is required for neovascularisation and may create favourable conditions for increased growth, but it is likely that other factors are required for tumour enlargement or inhibiting increased growth. Turner *et al.* observed that microprolactinomas are significantly less vascular than macroprolactinomas,

although they did not observe this relationship in GH-secreting tumours (47). Along with its association with VEGF, IL6 is likely to have other roles in the pathogenesis of these tumours. We know that the presence of IL6 is positively associated with pituitary tumour invasiveness (48, 49), and driving increased vascularisation would facilitate this process.

We do not know whether the increase in VEGF levels seen in response to exogenous IL1 α in both the cell-line and primary cultures is controlled by the same mechanism as autocrine stimulation. Nor do we know whether the association between basal IL1 α and VEGF requires IL6. However, we have already shown that basal IL1 α and IL6 secretions are significantly and positively associated (SA Borg, KE Kery, JA Royds, RD Battersby and TH Jones, unpublished observations). FS cells are a potential source of IL1 α , and while they are known to secrete IL6, it has not been reported that they secrete IL1 α . FS cells are uncommon within an adenoma, and it has been observed that a transition zone rich in FS cells exists between the adenoma and normal tissue (50, 51). Lohrer *et al.* (35) proposed that in addition to any other effects IL6 may have, it may enhance the release of VEGF from FS cells in the transition zone, further increasing levels of VEGF in the region.

In conclusion, we have shown that human pituitary tumours, independently of subtype, and HP75 cells constitutively secrete VEGF *in vitro*. There is a strong positive correlation between basal production of IL6, IL1 α and VEGF. Furthermore, exogenous IL1 α stimulates VEGF production *in vitro*. These findings demonstrate that cytokine production is closely linked to VEGF release. The mechanism of this association is not clear, as VEGF is not associated with tumour volume or invasive status. However, since IL6 is a marker of invasiveness and IL6 secretion was found here to be associated with tumour volume, it would be reasonable to assume that VEGF is required for vascularisation of the tumour under these circumstances, and that would explain the relationship of these cytokines to VEGF.

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