Neuroendocrinology Letters No.4 August Vol.26, 2005
 ISSN 0172–780X **www.nel.edu**

ORIGINAL ARTICLE

Stimulatory effect of GH3 cell line conditioned medium on the proliferation of the endothelial cell line (HECa10) *in vitro*

Hanna Ławnicka [1] & Jolanta Kunert-Radek [2]

[1] Department of Experimental Endocrinology and Hormone Diagnostics,
[2] Clinic of Endocrinology, Institute of Endocrinology, Medical University of Lodz, Lodz, Poland

Correspondence to: Hanna Ławnicka
Department of Experimental Endocrinology and Hormone Diagnostics
Institute of Endocrinology
Medical University of Lodz, 91-425 Lodz
3 Dr Sterling Street, Poland,
TEL/FAX: +48 (42) 6365427
EMAIL: hlawnicka@wp.pl

Submitted: January 18, 2005 *Accepted: February 17, 2005*

Key words: **angiogenesis; endothelial cells; pituitary tumour cell culture; GH3 cells; tumour conditioned medium; VEGF**

Neuroendocrinol Lett 2005; **26**(4):413–418 **PMID:** 16135999 NEL260405A21 ©Neuroendocrinology Letters **www.nel.edu**

Abstract

OBJECTIVES: The process of neoangiogenesis plays a key role in development and progression of solid tumours. The formation of new blood vessels different from the portal network system is important for pituitary tumour formation. The pivotal role of activated endothelial cells in angiogenesis was well documented. The degree of endothelial cells stimulation depends on the angiogenic activity of the tumorous cells.
The aim of the study was to elucidate the angiogenic properties of the lactosomatotrophic pituitary cell line.
MATERIAL AND METHODS: The study was carried out on the mouse endothelial HECa10 cell line subjected to medium obtained from the culture of rat lactosomatotrophic pituitary GH3 cell line. The endothelial cell growth was measured using the modified colorimetric method EZ4Y detecting the viable cells. The secretion of vascular endothelial growth factor (VEGF) to GH3 cells conditioned medium was assesed by immunoenzymatic method.
RESULTS: It was shown that the exposure of endothelial cells to pituitary tumorous conditioned medium significantly stimulated the cellular growth after 24 and 48 hours of incubation. Moreover, the concentration of VEGF in GH3 conditioned medium occurred to be 13 times higher than in the control medium.
CONCLUSION: Our results indicate the angiogenic activity of lactosomatotroph GH3 cell line and suggest the role of VEGF in pituitary tumorous angiogenesis.

Abbreviations:

bFGF	basic fibroblast growth factor
C–F10	complete F10 medium
C–RPMI	complete RPMI medium
GH	growth hormone
GH3–CM	GH3 conditioned medium
HUVEC	human umbilical endothelial cells
IGF–1	insulin-like growth factor
OD	optical density
PRL	prolactin
TCM	tumour conditioned medium
VEGF	vascular endothelial growth factor

Introduction

Angiogenesis, the development of new blood vessels is well known to be a crucial process for variety of physiological and pathological events [46]. Since Folkman et al [21] suggested the role of angiogenesis in tumour development, this process has been confirmed to play a key role in formation and progression of solid tumours. The molecular mechanisms of angiogenesis in malignant tumours is the result of the so-called angiogenic phenotype of neoplastic cells. It results in the excessive production of angiogenic growth factors stimulating the migration and proliferation of endothelial cells [49].

It was shown that the proliferation rate was 30–45 times grater in the endothelium of blood vessels in tumours than in normal vessels [10, 14, 15, 54]. The potential doubling time of the tumour endothelium was significantly shorter than the turnover time of the normal endothelial cells as well [27]. Nowadays there is a growing evidence showing the molecular differences between the vascular phenotype of normal and tumorous endothelium [13, 23]. Lane et al observed induction of special chemokines involved in neovascularization by Kaposi's sarcoma-associated herpes virus infected endothelial cells [34]. The strong expression of the specific growth factor by the majority of the glioblastoma cells as well as by vascular endothelial glioblastoma cells was stated [33]. The autocrine and paracrine stimulation of endothelium in tumorous angiogenesis was postulated. It was also demonstrated that invasive breast cancer vessels expressed proteins characteristic of proliferating and migrating endothelial cells and modulating endothelial apoptosis *in vitro* [42]. Therefore, endothelial cells are subjected to numerous neoangiogenesis studies and they occured to be a suitable experimental model useful for studies concerning the mechanisms of angiogenesis.

The studies on angiogenesis in pituitary adenomas revealed contradictory results. Some observations showed that pituitary adenomas had significantly lower vascular densities as compared to non- tumorous adenohypophyses [16, 31, 55]. On the other hand, increased vascularization was observed in experimentally induced pituitary tumours. The implantation of diethylstilbestrol resulted in marked hyperprolactinaemia and hyperplasia of pituitary prolactin cells accompanied by the increase of the pituitary vessel area and vessel density in rats [43, 44, 48]. The autopsy angiographic studies revealed the presence of extraportal artery supplies in 66% patients with microadenomas [25]. Pawlikowski observed the higher vascular density in human gonadotropinomas [45]. Vidal et al demonstrated a tendency of greater vascularization of invasive pituitary tumours [56]. In studies on lactotroph tumours, the invasive prolactinomas and macroprolactinomas appeared to be significantly more vascularized tumours [52, 53]. In the human pituitary tumours immunucytochemical studies of the basement membrane revealed changes characteristic of active arterial neovascularization as lack or fragmentation of this membrane [18].

The aim of the present study was to investigate some angiogenic properties of the pituitary lactosomatotrophic GH3 cell line by evaluation the effect of GH3 conditioned medium on the proliferation of cultured HECa10 endothelial cells.

Material and methods

The murine endothelial cell line HECa10 [8] and the rat pituitary lactosomatotrophic cell line GH3 [50] were used in the experiments.

Cell culture of endothelial HECa10 cells: The continuous culture of the cells was maintained in culture flasks (Nunc EasY flask 25cm^2, NUNC). The cells were cultured in the complete medium composed of RPMI 1640 medium (Sigma) supplemented with 25 mM Hepes buffer (Sigma); 2 g/liter sodium bicarbonate (Sigma); 50 μM 2–mercaptoethanol (Serva); 4 mM L–glutamine

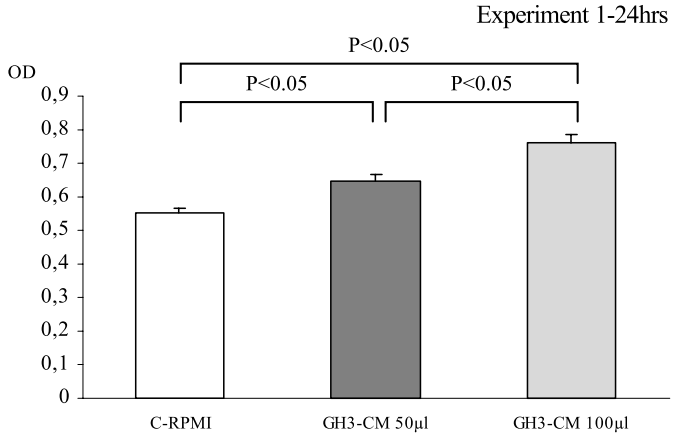

Figure 1A: The effect of GH3 conditioned medium (GH3–CM) on the viability of mouse endothelial cells (HECa10) incubated for 24 hrs; C–RPMI - control with complete RPMI, OD–optical density.
Bars represent means ± SEM

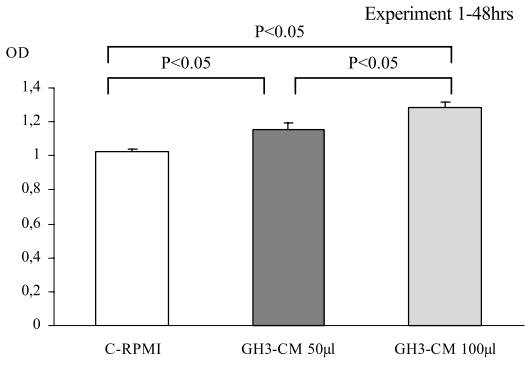

Figure 1B: The effect of GH3 conditioned medium (GH3–CM) on the viability of mouse endothelial cells (HECa10) incubated for 48 hrs; C-RPMI – control with complete RPMI, OD-optical density. Bars represent means±SEM

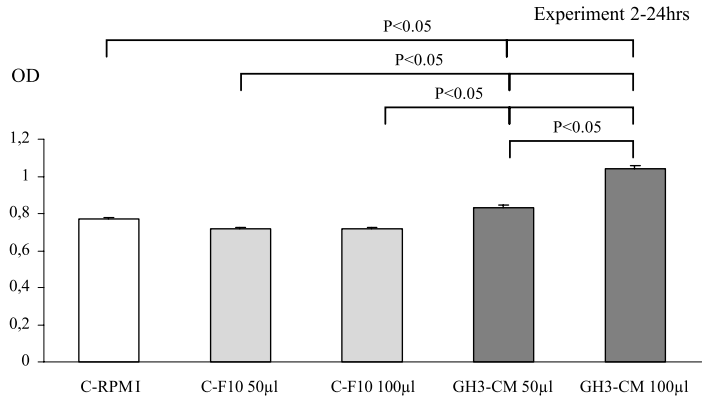

Figure 2A: The effect of GH3 conditioned medium (GH3–CM) on the viability of mouse endothelial cells (HECa10) incubated for 24 hrs; C/RPMI – control with complete RPMI; C-F10 – control with complete F-10, OD – optical density. Bars represent means±SEM.

(Sigma); 0.1 mM sodium pyruvate (Sigma); 100 U/ml penicillin and 100 µg/ml streptomycin solution (Sigma); 10% heat-inactivated fetal calf serum (FCS, Biochrom) at 37°C in the humidified atmosphere of 95% air and 5% CO2. Before confluency (twice a week) the cells were harvested after a 2–min incubation at room temperature in the presence of trypsin–EDTA (0.05 and 0.02% respectively) in Hanks-balanced salt solution (Sigma). The cells were washed three times in complete RPMI and after last centrifugation seeded at $5x10^4$ cells in 5ml of fresh medium.

Cell culture of lactosomatotrophic GH3 cells: The continuous culture of the cells was maintained in culture flasks (Nunc EasY flask 25cm^2, NUNC). The cells were cultured in complete medium composed of F–10 medium (Sigma) supplemented with 2 g/liter sodium bicarbonate (Sigma); 100 U/ml penicillin and 100 µg/ml streptomycin solution (Sigma); 2.5% heat-inactivated fetal calf serum (FCS, Biochrom) and 15% heat-inactivated horse serum (Sigma) at 37°C in the humidified atmosphere of 95% air and 5% CO2. According to the amount of cells the complete medium was changed every 3–4 days. About once a week, prior to confluency, the cells were harvested after a 2–min incubation at 37°C in the presence of trypsin-EDTA (0.05 and 0.02% respectively) in Hanks-balanced salt solution (Sigma). The cells were washed twice in complete medium and after last centrifugation seeded at 5x105 cells in 5ml of fresh medium. The supernatant obtained from culture flasks before harvesting GH3 cells (GH3–conditioned medium – GH3–CM) was collected and stored at –80°C.

Experiments: Two experiments were carried out. The HECa10 cells were subjected to the trypsinization process as described above, suspended at $1x10^5$/ml cells (experiment 1) and at $1.4x10^5$/ml cells (experiment 2) in complete RPMI and 50 µl aliquots of cell suspension ($5x10^3$ cells for experiment 1 and $7x10^3$ cells for experiment 2) were placed in the assay wells of cell culture plates (Nunclon^{TM} Δ 96 MicroWell Plates, NUNC). Then 50 µl or 100 µl of just defrosted GH3 conditioned supernatant (GH3–CM) was added to the appropriate wells. The equal volume of culture medium: complete RPMI (C–RPMI) for experiment 1 or complete RPMI and complete F–10 (C–F10) for experiment 2 was added to the control wells. The final volume in each well was 200 µl. After 24hrs or 48hrs of incubation the cell viability was measured using EZ4Y system (based on the reduction of substrate [3–(4,5–dimethylthiazol–2–yl)–2,5–diphenyl–tetrazolium bromide] to a formazan product by mitochondrial dehydrogenases in living cells), following the procedure recommended by the producer of the kit (EZ4Y, Easy for You, The 4th Generation Non Radioactive Cell Proliferation & Cytotoxity Assay, Biomedica Gruppe, Austria, Bellco Biomedica Poland). The optical density (OD), reflecting the quantity of viable cells in the sample was measured by microplate ELISA reader at 450 nm. In our previous studies a linear relationship between the OD and the number of viable HECa10 cell has been demonstrated (data not shown).

***VEGF concentration assay*:** Mouse vascular endothelial growth factor kit (VEGF Immunoassay kit, Quantikine M, R&D Systems, USA) was used to measure VEGF concentration in the GH3 conditioned supernatant (sensitivity<3pg/ml; intraassay precision CV<8%). The optical density (OD) of each sample was measured by microplate reader at 450 nm and the sample values of VEGF (pg/ml) were read off standard curve.

***Statistical evaluation*:** The results are expressed as means±SEM. Comparisons between tested groups were made by one-way analysis of variance. When ANOVA revealed a statistically significant difference, comparisons were further evaluated by the least statistical difference test (LSD). The differences were considered significant if P<0.05.

Results

In both experiments the exposure of HECa10 cells to GH3 conditioned medium (GH3–CM) significantly stimulated in a dose dependent way the growth of endothelial cells (*Figure: 1A, 2A, 1B, 2B*).

The concentration of VEGF in GH3 conditioned medium (GH3–CM) was 13 times higher as compared with its concentration in the control medium (10.02 vs 138.8 pg/ml, *Figure 3*).

Discussion

The observed stimulatory effect of GH3 conditioned medium on the proliferation of endothelial cells indicate some angiogenic activity of tumorous pituitary cells. These results are in agreement with some observations of other authors. In vivo the increased numbers of circulating endothelial cells in cancer patients was noticed [7]. In vitro the greater proliferative activity of endothelial cells incubated with tumour conditioned medium (TCM) obtained from breast carcinoma and from small lung carcinoma cell lines was shown [26]. Li et al who also examined the effect of breast carcinoma cell line conditioned medium on the cell cycle of human umbilical endothelial cells (HUVEC), revealed more endothelial cells in the S phase [35]. The mitogenic and anti-apoptotic activities of above medium on these endothelial cells were also observed [36]. The authors suggest that the breast carcinoma cell line may secrete soluble pro-angiogenic factors that induce the HUVEC angiogenic switch including cell cycle progression, proliferation and growth. Increased survival accompanied by some crucial for angiogenesis phenotypic changes of endothelial cells incubated with malignant oseoblastic cell line conditioned medium but without inducing the proliferation was observed by Castilla et al [12]. These effects occurred to be inhibited by the blockade of vascular endothelial growth factor (VEGF) or by blockade of VEGF receptor. VEGF is endothelial-cell-specific mitogen secreted by tumour tissue [1, 19, 51]. In our study VEGF produced by GH3 cells (*Fig. 3*) may be one of factors responsible for "angiogenic switch" and for the proliferative effect of GH3–CM on HECa10 cells. It is worth mentioning that whereas in normal pituitary VEGF has been reported to be produced only by folliculo-stellate cells [20, 30], pituitary tumour cells acquire the ability to produce VEGF. It was shown that both human pituitary tumour cells and different pituitary cell lines (GH3, AtT20, αT3–1) secreted VEGF under basal and stimulated conditions and that these tumorous pituitary cells might be involved in the angiogenic process in pituitary [4, 5, 37]. Our results are in agreement with these observations. The next possible mechanism is that GH3–CM caused stimulation of VEGF autocrine secretion by endothelial cells. Olaso et al observed the stimulation VEGF mRNA and protein synthesis by hepatic stellate cell line incubated with melanoma supernatants [41]. Autocrine secreted VEGF may additionally stimulate the proliferation of endothelial cells.

Boyd et al, who studied the cross talk between melanoma and endothelial cells in an *in vitro* co-culture model, revealed that uveal melanomas could support the growth of endothelium and that this effect could be modulated both by VEGF and by basic fibroblast growth factor (bFGF) [9]. In another study Il–8 produced by hepatocellular carcinoma cell line significantly accelerated the proliferation of HUVEC in co-culture of endothelial and carcinoma cells [2]. It is possible that not only VEGF but also bFGF or other cytokines are included in the observed in our study stimulatory effect of GH3–CM on HECa10 cells. This possibility can be supported by the observations of the elevated levels of VEGF and bFGF in the peripheral blood of patients with pituitary adenomas [32]. Induction of angiogenic phenotype through bFGF pathway by pituitary tumor-transforming gene (PTTG), protooncogene expressed in pituitary tumours [28], can also support above conclusion [58]. Moreover, medium conditioned from transfectants overexpressing the wild-type human

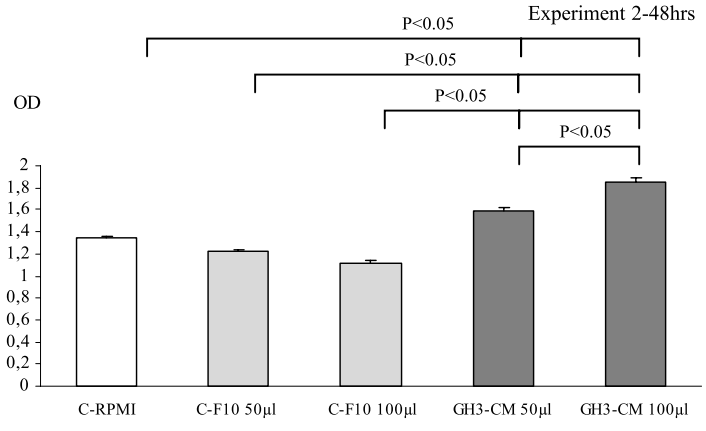

Figure 2B: The effect of GH3 conditioned medium (GH3-CM) on the viability of mouse endothelial cells (HECa10) incubated for 48 hrs; C-RPMI – control with complete RPMI; C-F10 – control with complete F-10, OD-optical density. Bars represent means±SEM

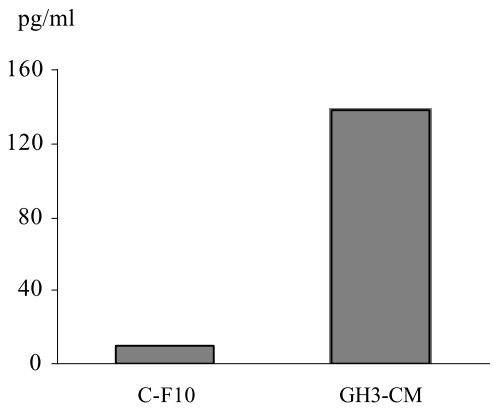

Figure 3: Concentration of VEGF (pg/ml) in complete F-10 medium (C-F10) and in GH3 conditioned medium (GH3-CM).

PTTG induced proliferation, migration and tube formation of endothelial cells [29].

The rat lactosomatotrophic cell line GH3 is known to secrete both prolactin (PRL) and growth hormone (GH) [3]. It has been shown that PRL posses both angiogenic and antiangiogenic properties. While in some studies the inhibition of endothelial cells proliferation and antiangiogenic effects of the 16–kDa fragment of PRL were shown [22, 24], the study of Malaguarnera et al demonstrated the stimulation of human endothelial cell proliferation and increase of capillary formation induced by prolactin [39]. Enhanced release of VEGF from PRL treated macrophages was also shown [38]. The ability of human prolactin antagonist to inhibit endothelial cell proliferation, to disrupt the tube structures formation and to inhibit the signal transduction induced by PRL in human breast cancer cells were shown as well [6]. In our study PRL secreted by GH3 cells may be involved in the stimulatory effect of GH3–CM on HECa10 cells. Growth hormone is another hormone secreted by GH3 cells. Raccurt et al localized the GH gene expression in surgical specimens of the mammary gland proliferative disorders. The authors detected GH mRNA in the endothelial cells in areas of neovascularization and suggested the pivotal role of autocrine GH in neoplastic mammary gland progression [47]. In our study the stimulatory effect of GH secreted by GH3 cells on endothelial cells proliferation may be also taken into consideration.

In our experiments the mechanism of apoptosis should also be discussed. The resistance of endothelial cells co-cultured with glioblastoma to apoptosis was shown [11]. According to the authors, the endothelial genes associated with survival may be up-regulated in endothelial cells growing in the tumour microenvironment. The ability of TCM to delay cell apoptosis was also shown by Wu et al [60]. The technique used in our study provides the global information on the number of living active cells. It is well correlated with other proliferation indices but it does not differentiate between the effects depending on proliferation, necrosis and apoptosis [40]. It is possible that the stimulatory effect of GH3–CM on the quantity of viable endothelial cells depends not only on the increased proliferation but on the inhibited apoptosis as well. Moreover, the observation by Wilson et al [59] that insulin-like growth factor (IGF–1) protected human retinal endothelial cells from apoptosis as well as the statement by Fagin et al [17] that GH3 cells expressed the gene for IGF–1 and secreted IGF–1 into the culture medium both may support that possibility.

Although pituitary adenomas seemed to be less vascularized than normal pituitary gland, the formation of new blood vessel network system, different from that portal one, remains crucial for pituitary tumour formation. The presence of specific Flk–1 receptors for the most potent angiogenic factor VEGF in endothelial cells of capillaries in the pituitary gland support above thesis [57].

In conclusion our results indicate the angiogenic activity of lactosomatotrophic GH3 cell line and suggest the role of tumourous VEGF in this process. Further studies are needed to explain the complete mechanism of this activity and for better understanding the pathogenesis of pituitary adenomas.

Acknowledgements

This study was supported by a grant from Medical University of Lodz (502–11–807).

REFERENCES

1 Affara NI, Robertson FM. Vascular endothelial growth factor as a survival factor in tumor-associated angiogenesis. In Vivo. 2004; **18**(5):525–42.
2 Akiba J, Yano H, Ogasawara S, Higaki K, Kojiro M. Expression and function of interleukin–8 in human hepatocellular carcinoma. Int J Oncol. 2001; **18**(2):257–64.
3 Bancroft FC, Tashjian AH Jr. Growth in suspension culture of rat pituitary cells which produce growth hormone and prolactin. Exp Cell Res. 1971; **64**(1):125–8.
4 Banerjee S, Saxena N, Sengupta K, Banerjee SK. 17alpha–estradiol–induced VEGF–A expression in rat pituitary tumor cells is mediated through ER independent but PI3K–Akt dependent signaling pathway.: Biochem Biophys Res Commun. 2003; **300**(1): 209–15.
5 Banerjee SN, Sengupta K, Banerjee S, Saxena NK, Banerjee SK. 2–Methoxyestradiol exhibits a biphasic effect on VEGF–A in tumor cells and upregulation is mediated through ER–alpha: a possible signaling pathway associated with the impact of 2–ME2 on proliferative cells. Neoplasia. 2003; **5**(5):417–26.
6 Beck MT, Chen NY, Franek KJ, Chen WY. Prolactin antagonist–endostatin fusion protein as a targeted dual-functional therapeutic agent for breast cancer. Cancer Res. 2003; **63**(13):3598–604.
7 Beerepoot LV, Mehra N, Linschoten F, Jorna AS, Lisman T, Verheul HM et al. Circulating endothelial cells in cancer patients do not express tissue factor. Cancer Lett. 2004;**213**(2):241–8.
8 Bizouarne N, Denis V, Legrand A, Monsigny M, Kieda C. A SV–40 immortalized murine endothelial cell line from peripheral lymph node high endothelium expresses a new α–L–fucose binding protein. Biol Cell. 1993; **79**(3):209–218.
9 Boyd SR, Tan DS, de Souza L, Neale MH, Myatt NE, Alexander RA et al. Uveal melanomas express vascular endothelial growth factor and basic fibroblast growth factor and support endothelial cell growth. Br J Ophthalmol. 2002; **86**(4):440–7.
10 Brien SE, Zagzag D, Brem S. Rapid in situ cellular kinetics of intracerebral tumor angiogenesis using a monoclonal antibody to bromodeoxyuridine. Neurosurgery. 1989; **25**(5):715–9.
11 Brown CK, Khodarev NN, Yu J, Moo–Young T, Labay E, Darga TE et al. Glioblastoma cells block radiation-induced programmed cell death of endothelial cells. FEBS Lett. 2004; **565**(1–3):167–70.
12 Castilla MA, Neria F, Renedo G, Pereira DS, Gonzalez–Pacheco FR, Jimenez S et al. Tumor-induced endothelial cell activation: role of vascular endothelial growth factor. Am J Physiol Cell Physiol. 2004; **286**(5):C1170–6. Epub 2004 Jan 07.
13 Demeule M, Regina A, Annabi B, Bertrand Y, Bojanowski MW, Beliveau R. Brain endothelial cells as pharmacological targets in brain tumors. Mol Neurobiol. 2004; **30**(2):157–83.
14 Denekamp J, Hobson B. Endothelial-cell proliferation in experimental tumours. Br J Cancer. 1982; **46**(5):711–20.
15 Denekamp J. Endothelial cell proliferation as a novel approach to targeting tumour therapy. Br J Cancer. 1982; **45**(1):136–9.
16 Erroi A, Bassetti M, Spada A, Giannattasio G. Microvasculature of human micro– and macroprolactinomas. A morphological study. Neuroendocrinology. 1986; **43**(2):159–65.
17 Fagin JA, Pixley S, Slanina S, Ong J, Melmed S. Insulin-like growth factor I gene expression in GH3 rat pituitary cells: messenger ribonucleic acid content, immunocytochemistry, and secretion. Endocrinology. 1987; **120**(5):2037–43.
18 Farnoud MR, Lissak B, Kujas M, Peillon F, Racadot J, Li JY. Specific alterations of the basement membrane and stroma antigens in human pituitary tumours in comparison with the normal anterior pituitary. An immunocytochemical study. Virchows Arch A Pathol Anat Histopathol 1992; **421**(6):449–55.
19 Ferrara N, Davis–Smyth T. The biology of vascular endothelial growth factor. Endocr Rev 1997; **18**(1):4–25.

20 Ferrara N, Henzel WJ. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Commun 1989; **161**(2):851–8.
21 Folkman J, Long DM, Becker FF. Growth and metastasis of tumor in organ culture. Cancer 1963; **16**:453–467.
22 Galfione M, Luo W, Kim J, Hawke D, Kobayashi R, Clapp C et al. Expression and purification of the angiogenesis inhibitor 16–kDa prolactin fragment from insect cells. Protein Expr Purif 2003; **28**(2): 252–8.
23 Giordano F, Mitola S. Characterization of endothelial cells isolated by human meningiomas. J Neurosurg Sci 2000; **44**(4):177–85.
24 Gonzalez C, Corbacho AM, Eiserich JP, Garcia C, Lopez–Barrera F, Morales–Tlalpan V et al. 16K–prolactin inhibits activation of endothelial nitric oxide synthase, intracellular calcium mobilization, and endothelium-dependent vasorelaxation. Endocrinology 2004; **145**(12):5714–22.
25 Gorczyca W, Hardy J. Microadenomas of the human pituitary and their vascularization. Neurosurgery. 1988; **22**(1 Pt 1):1–6.
26 Hewett PW. Identification of tumour-induced changes in endothelial cell surface protein expression: an in vitro model. Int J Biochem Cell Biol 2001; **33**(4):325–35.
27 Hobson B, Denekamp J. Endothelial proliferation in tumours and normal tissues: continuous labelling studies. Br J Cancer 1984; **49**(4):405–13.
28 Hunter JA, Skelly RH, Aylwin SJ, Geddes JF, Evanson J, Besser GM et al. The relationship between pituitary tumour transforming gene (PTTG) expression and in vitro hormone and vascular endothelial growth factor (VEGF) secretion from human pituitary adenomas. Eur J Endocrinol 2003; **148**(2):203–11.
29 Ishikawa H, Heaney AP, Yu R, Horwitz GA, Melmed S. Human pituitary tumor-transforming gene induces angiogenesis. J Clin Endocrinol Metab 2001; **86**(2):867–74.
30 Jabbour HN, Boddy SC, Lincoln GA. Pattern and localisation of expression of vascular endothelial growth factor and its receptor flt–1 in the ovine pituitary gland: expression is independent of hypothalamic control. Mol Cell Endocrinol 1997; **134**(2):91–100.
31.Jugenburg M, Kovacs K, Stefaneanu L, Scheithauer BW. Vasculature in Nontumorous Hypophyses, Pituitary Adenomas, and Carcinomas: A Quantitative Morphologic Study. Endocr Pathol 1995; **6**(2):115–124.
32 Komorowski J, Jankiewicz J, Stepien H. Vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and soluble interleukin–2 receptor (sIL–2R) concentrations in peripheral blood as markers of pituitary tumours. Cytobios 2000; **101**(398):151–9.
33 Kunkel P, Muller S, Schirmacher P, Stavrou D, Fillbrandt R, Westphal M et al. Expression and localization of scatter factor/hepatocyte growth factor in human astrocytomas. Neuro-oncol 2001; **3**(2):82–8.
34 Lane BR, Liu J, Bock PJ, Schols D, Coffey MJ, Strieter RM et al. Interleukin–8 and growth-regulated oncogene alpha mediate angiogenesis in Kaposi's sarcoma. J Virol. 2002; **76**(22):11570–83.
35 Li A, Li H, Jin G, Xiu R. A proteomic study on cell cycle progression of endothelium exposed to tumor conditioned medium and the possible role of cyclin D1/E. Clin Hemorheol Microcirc 2003; **29**(3–4):383–90.
36 Li A, Li H, Zhang J, Jin G, Xiu R. The mitogenic and anti-apoptotic activity of tumor conditioned medium on endothelium. Clin Hemorheol Microcirc 2003; **29**(3–4):375–82.
37 Lohrer P, Gloddek J, Hopfner U, Losa M, Uhl E, Pagotto U et al. Vascular endothelial growth factor production and regulation in rodent and human pituitary tumor cells in vitro. Neuroendocrinology 2001; **74**(2):95–105.
38 Malaguarnera L, Imbesi RM, Scuto A, D'Amico F, Licata F, Messina A et al. Prolactin increases HO–1 expression and induces VEGF production in human macrophages. J Cell Biochem 2004; **93**(1): 197–206.
39 Malaguarnera L, Pilastro MR, Quan S, Ghattas MH, Yang L, Mezentsev AV et al. Significance of heme oxygenase in prolactin-mediated cell proliferation and angiogenesis in human endothelial cells. Int J Mol Med 2002; **10**(4):433–40.
40 Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983; **65**(1–2):55–63.
41 Olaso E, Salado C, Egilegor E, Gutierrez V, Santisteban A, Sancho–Bru P et al. Proangiogenic role of tumor-activated hepatic stellate cells in experimental melanoma metastasis. Hepatology. 2003;**37**(3):674–85.
42 Parker BS, Argani P, Cook BP, Liangfeng H, Chartrand SD, Zhang M et al. Alterations in vascular gene expression in invasive breast carcinoma. Cancer Res 2004; **64**(21):7857–66.
43 Pawlikowski M, Grochal M, Kulig A, Zielinski K, Stepien H, Kunert–Radek J et al. The effect of angiotensin II receptor antagonists on diethylstilbestrol-induced vascular changes in the rat anterior pituitary gland: a quantitative evaluation. Histol Histopathol 1996; **11**(4):909–13.
44 Pawlikowski M, Kunert–Radek J, Grochal M, Zielinski K, Kulig A. The effect of somatostatin analog octreotide on diethylstilbestrol-induced prolactin secretion, cell proliferation and vascular changes in the rat anterior pituitary gland. Histol Histopathol 1997; **12**(4):991–4.
45 Pawlikowski M. Immunocytochemical prognostic markers in pituitary tumors. Folia Histochem Cytobiol 2001; **39**(2):105–6.
46 Pepper MS. Positive and negative regulation of angiogenesis: from cell biology to the clinic. Vascular Medicine. 1996; **1**(4):259–266.
47.Raccurt M, Lobie PE, Moudilou E, Garcia–Caballero T, Frappart L, Morel G et al. High stromal and epithelial human gh gene expression is associated with proliferative disorders of the mammary gland. J Endocrinol 2002; **175**(2):307–18.
48 Stepien H, Grochal M, Zielinski KW, Mucha S, Kunert–Radek J, Kulig A et al. Inhibitory effects of fumagillin and its analogue TNP–470 on the function, morphology and angiogenesis of an oestrogen-induced prolactinoma in Fischer 344 rats. J Endocrinol 1996; **150**(1):99–106.
49 Stepien HM, Kolomecki K, Pasieka Z, Komorowski J, Stepien T, Kuzdak K. Angiogenesis of endocrine gland tumours – new molecular targets in diagnostics and therapy. Eur J Endocrinol 2002;**146**(2):143–51.
50 Tashjian AH Jr, Yasumura Y, Levine L, Sato GH, Parker ML. Establishment of clonal strains of rat pituitary tumor cells that secrete growth hormone. Endocrinology 1968; **82**(2):342–52.
51 Toi M, Matsumoto T, Bando H. Vascular endothelial growth factor: its prognostic, predictive, and therapeutic implications. Lancet Oncol 2001; **2**(11): 667–73.
52 Turner HE, Nagy Z, Gatter KC, Esiri MM, Harris AL, Wass JA. Angiogenesis in pituitary adenomas – relationship to endocrine function, treatment and outcome. J Endocrinol 2000; **165**(2):475–81.
53 Turner HE, Nagy Z, Gatter KC, Esiri MM, Harris AL, Wass JA. Angiogenesis in pituitary adenomas and the normal pituitary gland. J Clin Endocrinol Metab 2000; **85**(3):1159–62.
54 Vartanian RK, Weidner N. Correlation of intratumoral endothelial cell proliferation with microvessel density (tumor angiogenesis) and tumor cell proliferation in breast carcinoma. Am J Pathol. 1994; **144**(6):1188–94.
55 Viacava P, Gasperi M, Acerbi G, Manetti L, Cecconi E, Bonadio AG et al. Microvascular density and vascular endothelial growth factor expression in normal pituitary tissue and pituitary adenomas. J Endocrinol Invest. 2003; **26**(1):23–8.
56 Vidal S, Kovacs K, Horvath E, Scheithauer BW, Kuroki T, Lloyd RV. Microvessel density in pituitary adenomas and carcinomas. Virchows Arch. 2001; **438**(6):595–602.
57 Vidal S, Lloyd RV, Moya L, Scheithauer BW, Kovacs K. Expression and distribution of vascular endothelial growth factor receptor Flk–1 in the rat pituitary. J Histochem Cytochem. 2002; **50**(4): 533–40.
58 Wang YK, Cui N, Li J, Luo B. Expression of pituitary tumor-transforming gene in endometrial carcinoma. Zhonghua Fu Chan Ke Za Zhi. 2004; **39**(8):538–42.
59 Wilson SH, Davis MI, Caballero S, Grant MB. Modulation of retinal endothelial cell behaviour by insulin-like growth factor I and somatostatin analogues: implications for diabetic retinopathy. Growth Horm IGF Res 2001; **11** Suppl A: S53–9.
60 Wu QD, Wang JH, Condron C, Bouchier–Hayes D, Redmond HP. Human neutrophils facilitate tumor cell transendothelial migration. Am J Physiol Cell Physiol 2001; **280**(4):C814–22.