CLINICAL STUDY

Circulating vascular endothelial growth factor and active renin concentrations and prostaglandin E₂ urinary excretion in patients with adrenal tumours

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Abstract

Objectives: The aim of the present study was to determine vascular endothelial growth factor (VEGF), prostaglandin E₂ (PGE₂) and active renin levels in patients with hormonally active adrenal tumours.

Design: The study was comprised of 16 patients with primary aldosteronism, 13 patients with active Cushing's syndrome due to adrenal adenomas, 8 patients with adrenal carcinomas, 19 patients with pheochromocytoma and 19 healthy volunteers.

Methods: Active renin in plasma was determined by a two-site immunoradiometric assay. VEGF in sera samples and PGE₂ in 24-h urine were measured by ELISA.

Results: VEGF was significantly elevated in all the four groups of patients as compared with the controls. VEGF levels in patients with Cushing's syndrome were higher than those in patients with primary aldosteronism. Patients with adrenal carcinomas had the highest VEGF levels and the differences reached significance as compared with patients with primary aldosteronism and pheochromocytoma. PGE₂ levels were not significantly different among groups. Active renin was significantly the lowest in patients with primary aldosteronism and significantly the highest in patients with pheochromocytoma compared with the controls. Active renin in patients with primary aldosteronism was significantly lower than in those with Cushing's syndrome, pheochromocytoma and adrenal carcinoma.

Conclusions: Our data indicated that the mean level of VEGF in patients with all investigated adrenal tumours was significantly higher than in healthy controls. The cortisol-producing tumours appear to have increased angiogenic potential. Angiogenesis is probably associated not only with malignancy but also with functional activity of the adrenal tumors.

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Introduction

Vascular endothelial growth factor (VEGF) plays an essential role in the initiation and regulation of the angiogenesis—biological process by which new blood capillaries are formed from pre-existing microvessels and venules. This action is associated with endothelial and tumour cell growth (1). Angiogenesis is essential for tumour growth and depends on production of angiogenic factors. VEGF mRNA was found to be expressed in tumour cells but not in endothelial cells, consistent with the hypothesis that VEGF is primarily a paracrine mediator (2). Furthermore, prognosis in patients with VEGF-positive tumours was worse than in those with VEGF-negative tumours (3). The interaction of VEGF with other potential angiogenic factors is not well established. Prostaglandins stimulate

angiogenesis but the precise mechanisms of their proangiogenic actions remain unexplained. In various cancer tissues prostaglandin E₂ (PGE₂) stimulates VEGF expression (4). On the other hand, the cyclooxygenase-2 (COX-2) inhibitor, NS-398, restores tumour cell apoptosis, reduces microvascular density and reduces tumour growth of PC-3 prostate carcinoma cells xenografted into nude mice (5).

The powerful capacity of VEGF to increase angiotensin converting enzyme (ACE) in endothelial cells suggests a synergistic relationship between VEGF and the renin–angiotensin system in vascular biology and pathophysiology (6). Recent reports suggest that angiotensin II (ATII) stimulates the synthesis and secretion of VEGF in human mesangial cells through activation of the ATII receptor type 1 (AT1) (7). The AT1 receptor plays an important role in ischaemia-induced angiogenesis

(8). There are few studies concerning the angiogenesis in adrenal tumours. The adrenal gland is a well vascularized organ and the role of VEGF in adrenal tumours is not clear (9).

The aim of the present study was to determine VEGF, PGE₂ and active renin levels in patients with hormonally active adrenal tumours - Cushing's syndrome, primary aldosteronism, pheochromocytoma and adrenal carcinoma.

Materials and methods

The study was comprised of 16 patients (9 females and 7 males, mean age 48.6 ± 2.15 years) with primary aldosteronism, 13 patients (9 females and 4 males, mean age 44.16 ± 2.88 years) with active Cushing's syndrome due to adrenal adenomas, 8 patients (6 females and 2 males, mean age 42.16 ± 2.67 years) with adrenal carcinomas, 19 patients (10 females and 9 males, mean age 42.2 ± 4.69 years) with pheochromocytoma and 19 healthy volunteers (10 females and 9 males, mean age 42.2 ± 2.59 years).

The diagnosis of primary aldosteronism was suspected in patients with: (i) spontaneous hypokalaemia (serum potassium concentration < 3.5 mmol/l); (ii) moderately severe hypokalaemia (serum potassium concentration < 3.0 mmol/l) while receiving conventional doses of diuretics; (iii) inappropriate kaliuresis (24-h urinary potassium value > 30 mmol) in the face of hypokalaemia (potassium concentration < 3.0 mmol/l); (iv) refractory hypertension. The diagnosis was based on elevated plasma aldosterone, low levels of plasma renin activity, failure of stimulation of $> 50\%$ of aldosterone levels during upright position and failure of suppression of $> 20\%$ of aldosterone secretion during a captopril test. The size and location of the aldosterone-producing adenomas were determined by computed tomography.

In the 13 patients with Cushing's syndrome adrenal adenomas were visualized. The diagnosis was based on typical clinical profile, elevated urinary excretion of cortisol, high plasma cortisol levels, loss of plasma diurnal cortisol rhythm, low level of adrenocorticotrophin (ACTH), and lack of suppression of plasma cortisol during low (2 mg) and high (8 mg) dose dexamethasone tests.

The diagnosis of pheochromocytoma was suspected on the basis of clinical characteristics including poorly controlled hypertension associated with bouts of sweating, headache and palpitations. It was confirmed when 24-h urinary catecholamine excretion exceeded 2–3 times the normal values. In all but one case the tumours were benign. Two patients from the group with pheochromocytomas were associated with neurofibromatosis type 1.

Six of the patients with adrenal carcinomas revealed signs of hypercortisolism, two of them showed cortisol and androgen hypersecretion and in one of them

catecholamines were elevated. Adrenal masses were visualized by computed tomography. In the patients with cortisol-secreting carcinoma, metastases in the liver were also detected. The smallest tumour size was observed in primary aldosteronism, whereas the greatest size was found in carcinomas.

The adrenal tumours in all patients were surgically removed. The diagnosis was additionally confirmed by histological investigation. Adrenal carcinomas were characterized by typical histological features (necrosis, haemorrhages, fibrosis or calcification, invasion of the adrenal capsule, blood vessel wall, or both, high mitotic rate, atypical mitoses, high nuclear grade, low percentage of clear cells) (9).

All patients and control subjects were included in the study only after giving informed consent. All investigations were performed at the time of diagnosis prior to the surgical treatment. All participants underwent complete clinical examination in order to exclude other pathology. Previous medication that could interfere with the measurement of investigated parameters was discontinued at least one week prior to the study.

Blood samples for active renin were taken in the morning after a 30-min rest in a sitting position and were collected in plastic tubes containing EDTA. Active renin was determined by two-site immunoradiometric assay (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA). The sensitivity of this assay as determined by the 95% confidence limit was 1.4 IU/ml. The monoclonal antibodies with high affinity and specificity to active renin were found to have a 0.2% cross reactivity with pro-renin. The intra- and interassay coefficients of variation were $< 2.5\%$ and $< 9.9\%$ respectively. VEGF in sera samples and PGE₂ in 24-h urine samples were measured by ELISA (R&D, Minneapolis, MN, USA). The intra- and interassay coefficients of variation for VEGF were 4.5% and 6.2% respectively and for prostaglandin E₂ were 5.8% and 8.9% respectively. All samples were run in the same assay to avoid interassay variations.

Statistical methods

The data were analysed using SPSS 11 software (SPSS, Chicago, IL, USA). The average values of each variable were compared using analysis of variance. Nonparametric Wilcoxon Signed Ranks test was used for VEGF, PGE₂, and active renin levels because the data were not normally distributed after logarithmic transformation. All results were expressed as means $\pm$ S.E.M. Statistical significance was fixed at $P < 0.05$.

Results

All of the investigated parameters in the controls and patients are given in Table 1. There was no statistical difference in age between controls and patients with adrenal tumours. In all patients' groups both systolic and diastolic blood pressure levels were significantly

Table 1 Circulating VEGF and active renin concentrations, and urinary PGE₂ in the four groups of patients with adrenal tumours and in healthy controls.

Variables	Controls	Cushing's syndrome	Primary aldosteronism	Phaeochromocytoma	Adrenal carcinoma
Number	19	13	16	19	8
Age (years)	42.2±2.52	44.16±2.88	48.6±2.5	42.2±4.69	42.16±2.67
Female/males	10/9	9/4	8/6	10/9	6/2
Systolic BP (mmHg)	125.2±4.6	152.3±5.5**	164.9±5.8***	159.3±5.89***	155.6±6.1***
Diastolic BP (mmHg)	78.1±4.2	89±3.2**	97.7±3.5***	96.2±4.1***	92.2±3.56***
Tumour size	—	3.8±1.1	2.2±0.89	4.9±2.1	6.4±1.57†
VEGF (pg/ml)	287.4±22.3	1240.0±312.5**	520.2±69.1**	585.3±138.0*	1372.0±210.2***
PGE ₂ (ng/24 h)	320.74±43.41	309.77±68.9	290.4±72.1	324.03±41.0	350.13±60.0
Active renin (pg/ml)	25.09±5.79	75.3±25.4	6.67±2.3*	125.43±38.6*	60.3±19.2

BP, blood pressure.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the control group; † $P < 0.05$ compared with primary aldosteronism.

higher in comparison with the control group. However, blood pressure levels did not differ significantly among the different patients' groups. No significant correlation between the size of the tumours and the plasma VEGF levels was found ($r = 0.28$, $P > 0.05$). The tumours' mean size differed significantly only between patients with primary aldosteronism and those with adrenal carcinomas ($P < 0.05$).

VEGF was significantly elevated in all the four groups of patients compared with the controls. VEGF levels in the patients with Cushing's syndrome were higher than those in patients with primary aldosteronism ($P < 0.05$). Patients with adrenal carcinomas had the highest VEGF levels and the differences reached significance as compared with those in patients with primary aldosteronism ($P < 0.001$) and phaeochromocytoma ($P < 0.01$). Prostaglandin E₂ levels were not significantly different among groups. As expected, active renin was significantly the lowest in the patients with primary aldosteronism and significantly the highest in patients with phaeochromocytoma compared with the controls. The level of active renin in patients with primary aldosteronism was significantly lower compared with patients with Cushing's syndrome ($P < 0.05$), phaeochromocytoma ($P < 0.01$) and adrenal carcinoma ($P < 0.05$).

Discussion

The adrenal gland is a well-vascularized organ and the role of VEGF in adrenal tumorigenesis is not well characterized. Recent studies demonstrated that VEGF mRNA was constantly expressed in normal adrenal cells as well as in cultured adrenocortical cells (9). We investigated VEGF and PGE₂ levels that are considered markers of angiogenesis. Tumours originating from the adrenal cortex represent a heterogeneous group as regards their functional activity and malignancy. Some authors suggest a correlation between VEGF levels and the functional activity of the tumours (10). We found that in all our groups of patients with adrenal tumours the mean level of serum VEGF was significantly higher as compared with the control

patients. Moreover, in patients with Cushing's syndrome VEGF was significantly higher as compared with patients with primary aldosteronism. Our data are indirectly supported by *in vitro* studies demonstrating that VEGF mRNA levels are increased after 24-h stimulation with either ACTH or cAMP (10). On the other hand, in the study of Bernini *et al.* (11) cortisol-producing adenoma showed an angiogenic phenotype comparable to normal specimens, without correlation with cortisol and ACTH levels, and significantly higher VEGF expression was shown in aldosterone-producing adenomas than in normal cortex. The authors had measured intratumoral vascular density by immunocytochemistry using CD34, a marker of endothelial cells, and angiogenic status was investigated by calculation of VEGF expression. This might explain some differences between our results and theirs as we have measured serum VEGF levels and we do not have data concerning VEGF expression.

VEGF is expressed in phaeochromocytomas as well as in paragangliomas and may contribute to the extreme vascularity of these tumours (12). In human phaeochromocytomas, angiogenesis has been associated with tumour progression. Higher VEGF gene expression was found in malignant phaeochromocytomas (13, 14). VEGF-mediated angiogenesis was inhibited by anti-VEGF antibodies in experimental tumours (14). Recently, moderate to strong VEGF expression in malignant phaeochromocytomas, and negative or weak expression in benign adrenal phaeochromocytomas has been reported (15). In our study, we found that VEGF levels in patients with benign phaeochromocytomas were significantly higher than in the healthy controls but significantly lower compared with the levels in patients with adrenal carcinomas. Thus, our results indirectly support the above-mentioned data concerning the levels of VEGF in benign phaeochromocytomas.

Experimental and clinical data suggest that serum VEGF levels are significantly higher in patients with various forms of human cancer than in those with benign ones. Overexpression of VEGF has been reported in malignant adrenal tumours (10, 16). In the study of Bernini *et al.* adrenal carcinomas showed increased

VEGF expression but reduced vascularization compared with that observed in normal tissue and in benign adenomas (11). Despite the low vascularization, malignant tumours preserved their aggressiveness. The data of Ljungberg *et al.* are similar; they found that patients with renal cell carcinoma having lower VEGF(121) mRNA levels had significantly longer survival time compared with those with higher levels. There was an inverse relation between expression of VEGF mRNA and serum VEGF levels (17).

Thus, VEGF might be applied as a marker of the malignancy of the tumours. Therefore, VEGF was supposed to be of prognostic value in patients with such tumours. Our patients with malignant tumours also showed significantly higher VEGF levels compared with the control group and the groups with primary aldosteronism and pheochromocytoma. But VEGF levels in patients with adrenal carcinomas did not differ significantly compared with the patients with Cushing's syndrome. Here, we must emphasize that all patients with carcinomas showed functional activity of the adrenal glands and that the relationship between intratumoral and circulating concentrations of VEGF is unpredictable.

In various cancer tissues VEGF co-localizes with COX-2, the rate-controlling enzyme of prostaglandin synthesis. Strong COX-2 and VEGF expression highly correlated with increased tumour microvascular density; new vessels proliferate in areas of the tumours that express COX-2. *In vitro*, PGE₂ is an inducer of basic regulators of angiogenesis, including VEGF (5, 18). In our study, urinary PGE₂ did not differ significantly among the four groups of adrenocortical tumours, suggesting that PGE₂ might not be a relevant marker of adrenal tumours.

It has been suggested that renin angiotensin system (RAS) is involved in VEGF-mediated tumour development and angiogenesis. Angiotensin II plays a significant role in tumour angiogenesis and growth *in vivo* through the AT1 receptor pathway (19). *In vitro* study showed that the ACE inhibitor, perindopril, inhibited VEGF-induced endothelial cell migration and significantly attenuated VEGF-mediated tumour development (20). In other experimental models, ACE inhibitors augmented angiogenesis (21, 22), leaving the role of the RAS in angiogenesis unclear.

We aimed at comparing the effect of RAS on VEGF production in diseases with high, normal and low renin levels. VEGF levels in all the patients were higher in comparison with the healthy controls. No significant correlation between VEGF and active renin was established. Our data suggested that in the investigated patients with adrenal tumours characterized by different renin levels no direct relationship between RAS and VEGF exists. We must note that in healthy subjects there is a strong correlation between the levels of active renin and angiotensin II. Since hypercortisolism is associated with increased

production of angiotensinogen (23, 24), in this case circulating levels of angiotensin II do not directly reflect active renin levels. In our patients with Cushing's syndrome showing a trend to higher active renin levels, there is a possibility for the level of angiotensin II to be much increased. The latter might contribute to the elevated VEGF levels in these patients.

It has previously been shown that plasma VEGF was higher in hypertensive patients compared with controls (25, 26) and that the VEGF levels correlated significantly with age, systolic and diastolic blood pressure, 10-year cardiovascular disease (CVD) risk, and CVD risk scores. Indices of endothelial damage and angiogenesis are beneficially changed by intensive cardiovascular risk factor management (26). This fact raises the possibility of a link between abnormal angiogenesis and arterial hypertension. The patients included in our study had endocrine hypertension and an increased CVD risk that may contribute to the elevated VEGF levels.

In conclusion, our data indicated that the mean level of VEGF in all the patients with the investigated adrenal tumours was significantly higher than in healthy controls. The cortisol-producing tumours appear to have increased angiogenic potential. Our results also suggest that angiogenesis is associated not only with malignancy but also with functional activity of the adrenal tumors.

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