Evaluation of parathyroid gland angiogenesis in chronic kidney disease associated with secondary hyperparathyroidism

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Abstract
Background. Secondary hyperparathyroidism (SHPT) is a common complication of chronic kidney disease. Increased parathyroid hormone (PTH) synthesis and secretion is associated with parathyroid cell hyperplasia. The exact mechanisms involved in parathyroid gland (PTG) hyperplasia are still poorly understood. There is no available data on angiogenesis in PTG of patients with chronic kidney disease and SHPT. The aim of this study is to evaluate angiogenesis and expression of the angiogenic factors, basic fibroblastic growth factor (b-FGF) and vascular endothelial growth factor A (VEGF), in secondary PTG hyperplasia.

Methods. This study was performed on formalin-fixed paraffin-embedded archival tissues of 21 SHPT glands from haemodialysis (n = 19) and kidney transplanted (n = 2) patients submitted to surgical parathyroidectomy. For control, eight normal human parathyroid glands (NPG) encountered in surgical specimens of total thyroidectomy were used. We evaluated the immunohistochemical expression of the proliferation cell marker Ki67. Angiogenesis was evaluated by immunohistochemistry staining with anti-endoglin (CD105) antibody in 21 SPH and 5 NPG by stereological analysis. Levels of b-FGF and VEGF were determined by semi-quantitative analysis in 21 SPH and 8 NPG.

Results. The SHPT patients present a mean iPTH of 1314 ± 750 pg/ml, a corrected serum calcium of 10.3 ± 1.2 mg/dl and a serum phosphorus of 6.1 ± 1.4 mg/dl. SHPT glands displayed a significantly higher immunoreactivity against Ki67, compared to NPG. With CD105, a significantly higher number and volume of microvessels were observed in SHPT compared to NPG. Both VEGF and b-FGF expression were increased in SHPT compared to NPG. Using the predefined subdivision into negative and positive only the b-FGF expression was significantly increased in the SHPT glands compared to NPG.

Conclusion. These results suggest that PTGs in this group of patients with SHPT have a significantly higher number of vessels expressing CD105, which has been reported to preferentially label activated endothelial cells associated with angiogenesis. SHPT glands have a significantly increased expression of b-FGF compared to NPG. VEGF-A expression is also increased in the examined SHPT glands but could be less relevant for angiogenesis.

Keywords: angiogenesis; chronic kidney disease; parathyroid gland; secondary hyperparathyroidism

Introduction

The parathyroid tissue is characterized by a very low degree of cellular turnover. Parathyroid cellular lifespan has been evaluated to be 2 years in adult mice and 20 years in a human adult [1]. Under physiological conditions, similar rates of apoptosis and proliferation are expected to ensure organ homeostasis.

In patients with chronic kidney disease (CKD) stage V on haemodialysis, the attempts to characterize cellular proliferation rates using markers like the proliferation cellular nuclear antigen (PCNA) or Ki67 yielded heterogeneous results, with significant variation of the proliferative activity between several studies and also between several glands [2]. However, it has been clearly demonstrated that the increase in the size of the PTG, in uraemic patients, is a consequence of cellular proliferation rather than cellular hypertrophy [3,4].

The evaluation of the apoptosis rate is even more difficult, resulting from technical difficulties related to the identification of a very reduced number of cells undergoing apoptosis for each time point, in tissues with low remodelling [2].

Along with these parathyroid cell changes in CKD patients with SHPT, modulator effects originated in vascular tissue are likely to occur. Several lines of evidence favour such modulatory effect.

The implantation of the parathyroid tissue in the muscle structures of the forearm is a relatively common surgical procedure, and the implanted tissue has the capacity of developing its own vascularization and maintaining function. Saxe et al. [5] described the existence of angiogenesis...
in fragments of parathyroid tissue resulting from the adenomatous and hyperplastic human PTGs transplanted to the iris in rabbits. Using RT-PCR and SELDI techniques, an increase in the production of angiopoietin-2 and VEGF (vascular endothelial growth factor) has been reported in a parathyroid tissue explanted in a model of isolated rat microvessels. These authors found that the increase in the VEGF production was essential for the angiogenesis induced by the explanted PTG, even though the angiogenesis induced exceeded the levels expected for an isolated effect of the VEGF-A, suggesting that other factors could be implicated in the process.

One of those factors, basic fibroblast growth factor-2 (b-FGF-2), synergizes with VEGF in the endothelial cell culture [6]. Interestingly, the presence of b-FGF and its receptor has already been identified in parathyroid adenomas, in primary and secondary PTG hyperplasia [7].

D’Adda et al. showed that endothelial components of hyperplastic PTGs from six patients with primary hyperparathyroidism associated with MEN-1 syndrome were increased when compared to glands from six patients with SHPT associated with CKD [8]. Other study evaluated angiogenesis and lymphangiogenesis in adenomas and primary hyperplasia using CD34 and LYVE-1 in the vascular and lymphatic endothelium, respectively, as well as the angiogenic and lymphangiogenic growth factors, VEGF-A, VEGF-C, b-FGF. These results showed an increased angiogenesis in the proliferative lesions when compared to normal glands and a pro-angiogenic effect of the b-FGF in the parathyroid tissue [9].

There are limited data evaluating the angiogenic process of the parathyroid hyperplastic glands of CKD patients with SHPT. In particular, the role of endoglin in this setting is unknown, although its involvement in other angiogenic conditions was reported [10]. We sought to evaluate the angiogenesis of hyperplastic glands from CKD patients with SHPT in comparison to normal glands.

A modulatory effect originated in vascular tissue raises the possibility of using angiogenic inhibitors to prevent or induce apoptosis and gland involution by simply reducing the vascular support necessary for the maintenance of parathyroid tissue metabolic functions.

Material and methods

Tissue sampling and data collection

Specimens of PTGs from 21 chronic kidney disease patients on haemodialysis and 2 patients recipients of a cadaveric kidney transplant were studied. All these patients underwent sub-total parathyroidectomy for SHPT. One PTG from each patient was randomly chosen. All PTGs were formalin-fixed and paraffin embedded. Serial sections from paraffin blocks were stained with hematoxylin–eosin and the clinical diagnosis was confirmed by histological analysis. For each slide, two pathologists independently evaluated the type of hyperplasia.

For controls, tissue samples from eight normal human parathyroid glands (NPG) resected during a total thyroidectomy surgery were prepared.

Demographic, clinical and laboratorial data were obtained using a randomly, blinded retrospective analysis of chronic kidney disease patients’ medical records. Demographic and histological data from patients with NPGs were also recorded.

Immunohistochemistry

Parathyroid tissue immunohistochemistry was carried out in 4-µm-thick sections, using the detection system polymer, EnVision (Dako, Denmark) and the streptavidin–biotin–immunoperoxidase technique (Ultra Vision Large Volume Detection System Anti-Polyvalent HRP, Lab Vision Corp, Fremont, USA) according to the manufacturer’s instructions. Antigen retrieval was performed with a Tris buffer, pH 6 for Ki67, with a ‘retrieval’ solution (Vector Laboratories, CA) for VEGF and b-FGF and with 3% pepsin in distilled water at 37°C for 30 min for CD105.

Endogenous peroxidase activity was blocked by incubating the sections with H2O2 in methanol for 10 min.

Unspecific binding was blocked by incubating the samples with Ultravision block (Lab Vision Corp., Fremont, USA) before the incubation with primary antibodies for CD105, VEGF-A and b-FGF.

Ki67 (NeoMarkers for Lab Vision Corp., Fremont, USA) was used at a dilution of 1:300 and incubated for 30 min.

The endothelial cell marker CD34 (Novocasta Laboratories, Newcastle, UK) was applied at a dilution of 1:40 and incubated for 30 min. Monoclonal mouse anti-human CD105 (DakoCytomation Inc., CA, USA) and VEGF-A mouse anti-vascular endothelial growth factor A (Zymed Laboratories Inc., CA, USA) were used at a dilution of 1:10 and 1:50 with incubation periods of 30 and 60 min, respectively. b-FGF (Transduction, Lexington, KY) was used at a dilution of 1:80 and incubated for 60 min. After final labelling with diaminobenzidine, the sections were washed in water, counterstained with Meyer’s hematoxylin for nucleus and mounted in a mounting medium (Richard-Allan Scientific, MI, USA).

As internal positive controls, eight parathyroid samples lacking histological changes were used in a tissue microarray (TMA). Representative areas were chosen from hematoxylin–eosin slides and placed in a recipient block with 23 holes.

Lymph node sections were used as internal positive controls for Ki67. Renal oncocytoma sections were used as positive controls for VEGF-A and b-FGF staining; breast carcinoma sections were used as positive controls for CD105 staining. Negative controls were obtained by omitting the primary antibodies.

Assessment of Ki67 staining

Ki67 labelling was studied in 21 SHPT and 8 NPG. Ki67 staining was expressed as the percentage of the total number of parathyroid cells and was assigned to one of five categories: 1, <1%; 2, 5–25%; 3, 25–50%; 4, 50–75% and 5, >75%. Less than five positive cells were used as a cut-off to define negative glands. A positive reaction was characterized by the presence of a distinct granular brown staining confined to the nucleus while the cytoplasm was unreactive.
Assessment of CD105 staining

Three random digital photographs with ×200 magnification were obtained from each sample for CD105-labelled sections [11]. Staining with CD105 was evaluated in tissue from 21 SHPT glands and 5 NPG.

Stereological analysis was performed measuring vascular volume and numerical density. Volume density ($V_v$) is the volume of a particular feature or structure in relation to the volume of the feature that contains it (for example, the volume of the nucleus in relation to the volume of the cell). Numerical density ($N_v$) is the number of features or particles present in a defined containing volume (for example, the number of cells in a defined volume of tissue).

To assess the numerical density ($N_v/V$) and volume density ($V_v/V$) of microvessels, a 0.84 mm$^2$ coherent stereological test system composed of 500 line intersects was super-imposed randomly over the image. A total area of 2.52 mm$^2$ and 1500 line intersects per sample was then studied. Selected microvessels were considered as those that had one endothelial cell layer, with no muscle layer [11].

Structures were counted in each of the intersects as follows: nucleus, cytoplasm, vessel and other structures (arteriole, venules and adipose tissue). Finally, total microvessels were also counted ($V$).

Volume density for each structure (i) was calculated as $V_{vi} = \sum j/1500$, where $\sum j$ is the total number of line intersections on the structure, and the microvessel numerical density ($N_v/V$) was calculated as $K/\beta(V_v/area,\sqrt{(V_v/area)/(V_v)^{1/2}})$ ($K = 1.07$, $\beta = 2.08$ and the area studied is 2.52 mm$^2$). The value for $\beta$ was calculated after a preliminary estimate of vessel shape provided 40% round, 40% elliptical and 20% cylindrical vessels [11].

Assessment of VEGF-A and b-FGF staining

b-FGF and VEGF labelling was studied in 21 SHPT and 8 NPG; staining was semiquantitatively assessed according to a previously defined scale: 0 (0 cells stained), 1 (<25% cells with mild staining), 2 (25–74% cells with moderate staining) and 3 (>75% cells with intense staining) and further divided into negative (0 and 1) and positive (2 and 3).

Statistics

Differences in stereological analysis for CD105 were compared using the Mann–Whitney test or Student’s t-test. Differences in VEGF-A and b-FGF expression were assessed by the chi-square analysis or Fisher’s exact test. A $P$-value $<0.05$ was accepted as statistically significant. Data are expressed as means ± SD values. Tests of variance homogeneity, normality and distribution were performed to ensure that the assumptions required for standard parametric analysis of variance were satisfied. In all analyses, the null hypothesis was rejected at the 0.05 level.

Results

Patients

Twenty-one patients with chronic kidney disease and secondary hyperparathyroidism were evaluated, 14 females and 7 males, with the mean age of 56.1 ± 10.8 years. Nineteen patients were on a regular haemodialysis program at the time of parathyroidectomy and two had been submitted to cadaveric kidney transplantation. One underwent two parathyroid surgeries in 14 months, the second due to recurrent secondary hyperparathyroidism. The time spent on dialysis program prior to parathyroidectomy was 106.1 ± 68.1 months. For kidney transplanted patients the time spent on dialysis and the time between the transplant surgery and parathyroidectomy was 269.5 and 50.2 months, respectively. The mean iPTH levels were 749.5 pg/ml (range: 300–3472 pg/ml). Albumin corrected serum calcium and phosphorus levels were 10.3 ± 1.2 and 6.1 ± 1.4 mg/dl, respectively. Nine patients were on active vitamin D therapy at date of parathyroidectomy.

As parathyroid controls, we studied eight glands incidentally removed from normocalcemic patients during thyroid surgery performed for multinodular goitre ($n=3$), colloid nodule ($n=1$), papillary carcinoma of the thyroid ($n=3$) and follicular carcinoma of the thyroid ($n=1$) in seven female patients and one male patient, with the mean age of 39.4 ± 12.0 years.

![Fig. 1](image-url) (A) Ki67 negative immunoreaction in tissue from NPG (left) and Ki67 positive, grade 5 immunoreaction in the adjacent lymph node tissue (right); (immunoperoxidase stain, ×400). (B) Ki67 positive, grade 2 immunoreaction in the parathyroid tissue from a patient with SHPT (immunoperoxidase stain, ×400).
*PTGs*

The histological evaluation of the parathyroid tissue resected from the 21 patients revealed multinodular hyperplasia in all except one patient that presented with diffuse hyperplasia. The mean weight of the glands was 1.46 ± 0.2 g.

The Ki67 expression was positive in all SHPT glands (68.2%, 27.3% and 4.5% assigned to categories 1, 2 and 3, respectively) and higher (P < 0.001) when compared to the negative stains of all NPGs (Figure 1).

With CD105, significantly higher numerical density and volume were observed in SHPT compared to NPG (NV vessels, 1352.7 versus 415.4 vessels/mm², P < 0.01; and VV vessels, 0.08 versus 0.02, P < 0.01). There were no differences in VN nucleus between the two groups. We also found a significant higher VN other structures in NPG, compared to SHPT (0.17 versus 0.02, P < 0.01) (Table 1). There was no association between vitamin D therapy and CD105 expression (Table 2).

VEGF-A positive cytoplasmatic staining was present in both SHPT and NPG, with a heterogeneous distribution in the parathyroid tissue.

Both VEGF and b-FGF expressions were increased in SHPT compared to NPG (Table 3). Using the predefined subdivision into negative and positive, only the b-FGF expression was significantly increased in the SHPT glands compared to NPG (Table 4).

The immunostainings are illustrated in Figure 2.

**Discussion**

Many studies have already shown that several angiogenic growth factors are produced and secreted by normal endocrine tissues and are increased in pathological conditions such as inflammation, hyperplasia and neoplastic disorders [12]. The pituitary adrenocorticotrophin hormone (ACTH) is the major trophic factor regulating and maintaining adrenocortical functions, affecting cell proliferation, migration and survival. The steroidogenic adrenal gland represents a tissue with an intense capillary network of highly permeable and fenestrated vessels. There are experimental data suggesting that the trophic effects of ACTH on the adrenal cortex are mediated, at least, in part via ACTH induction of VEGF-A expression by endocrine tissue that results in stimulation of vascularization [13].

To our knowledge there are very few data in parathyroid tissue angiogenesis from patients with chronic renal failure and secondary hyperparathyroidism. Our study showed that the PTGs of patients with SHPT have an increased number of neovessels combined with an increased expression of angiogenic factors VEGF-A and b-FGF compared to NPG.

Endothelial cells are highly heterogeneous cells and it has been suggested that pan-endothelial cell markers are not ideal markers for pathological or activated neovessels [12].

CD105 or endoglin is a type I trans-membrane protein highly expressed in human vascular endothelial cells. Endoglin binds specially to activate endothelial cells involved in angiogenesis. An up-regulation of endoglin expression in proliferating cells and in neoplastic vascular cells has already been shown. High levels of endoglin were detected in human microvascular endothelium and in tissues involved in active angiogenesis, as regeneration tissues, inflammatory and neoplastic tissue [10]. For these reasons we decided to perform immunohistochemistry with CD105 in PTGs. Our results showed that PTGs in this group of patients with SHPT have a significantly higher number of vessels expressing CD105, suggesting that PTG hyperplasia in secondary hyperparathyroidism is associated with increased angiogenesis.

The presence of adipose tissue in NPGs, contributing to 17% of the gland weight, is mainly responsible for differences observed in the volume density of other structures (arteriole, venules and adipose tissue) in the stereological analysis. The hyperplastic glands from chronic kidney disease patients with SHPT have a reduced amount of adipose tissue.

**Table 1.** CD105 stereological analysis (NV: numerical density; VV: volume density)

<table>
<thead>
<tr>
<th></th>
<th>SHPT (n = 22)</th>
<th>NPG (n = 5)</th>
<th>P</th>
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<tbody>
<tr>
<td>CD105</td>
<td></td>
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<tr>
<td>NV vessels (mean ± SD)</td>
<td>1352.0 ± 549.00</td>
<td>415.45 ± 267.5</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>VV vessels (mean ± SD)</td>
<td>0.08 ± 0.02</td>
<td>0.02 ± 0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>VN nucleus (mean ± SD)</td>
<td>0.30 ± 0.07</td>
<td>0.24 ± 0.04</td>
<td>NS</td>
</tr>
<tr>
<td>VN other (mean ± SD)</td>
<td>0.02 ± 0.04</td>
<td>0.17 ± 0.05</td>
<td>&lt;0.01</td>
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**Table 2.** Association between vitamin D and CD105 vessels numerical density (NVv)

<table>
<thead>
<tr>
<th></th>
<th>Vitamin D (n = 9)</th>
<th>No vitamin D (n = 13)</th>
<th>P</th>
</tr>
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<tr>
<td>NVv vessels (mean ± SD)</td>
<td>1309.62 ± 628.95</td>
<td>1414.84 ± 436.50</td>
<td>0.67</td>
</tr>
</tbody>
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**Table 3.** VEGF-A and b-FGF semi-quantitative grade

<table>
<thead>
<tr>
<th>VEGF-A*</th>
<th>SHPT glands (n = 22)</th>
<th>NPG (n = 8)</th>
<th>b-FGF**</th>
<th>SHPT glands (n = 22)</th>
<th>NPG (n = 8)</th>
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<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>3</td>
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<td>1</td>
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<tr>
<td>2</td>
<td>9</td>
<td>3</td>
<td>2</td>
<td>8</td>
<td>0</td>
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<tr>
<td>3</td>
<td>4</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>0</td>
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</table>

*P = 0.035.
**P = 0.031.

**Table 4.** VEGF and b-FGF positive and negative

<table>
<thead>
<tr>
<th></th>
<th>SHPT glands (n = 22)</th>
<th>NPG (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF* positive</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>VEGF negative</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>b-FGF** positive</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>b-FGF negative</td>
<td>10</td>
<td>8</td>
</tr>
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*P = 0.68.
**P = 0.03.
The VEGF-A is a highly conserved dimeric glycoprotein that belongs to the super-family of endothelial growth factors. VEGF-A has an important role in the physiology and pathophysiology of the angiogenic process. It increases microvessels’ permeability, stimulates the migration and division of endothelial cells, altering its genetic expression pattern and protecting them against apoptosis and cell senescence [13]. In this study we verified an increased expression of VEGF-A in the glands of SHPT patients. Although when we divided the groups into VEGF positive and VEGF negative, that difference does not have statistic significance.

It has been demonstrated in another study that the human parathyroid tissue is able to induce angiogenesis and that this phenomenon was dependent on a paracrine action of VEGF-A [14]. The parathyroid tissue stimulates VEGF-A mRNA synthesis, and the soluble type 1 VEGF receptor was able to completely block the vascular growth induced by the in vitro parathyroid tissue. The amount of angiogenesis induced by the parathyroid tissue exceeded the amount induced by VEGF-A, suggesting the existence of other factors accounting for this process [14]. A recent study [15] showed an increased angiogenesis in parathyroid adenomas compared to parathyroid proliferative lesions from patients with SHPT and an increased angiogenesis in the latter compared to normal glands. The lack of correlation between CD105 and VEGF expression suggests that VEGF must not be considered the primary pro-angiogenic factor in the parathyroid tissue.

The fibroblast growth factors (FGF) are a growth factor family with an important role in glandular tissue proliferation. The b-FGF is a potent angiogenic factor, stimulating the proliferation of endothelial cells. It is produced by endothelial, stromal and tumoural cells and it is also released by an extracellular matrix. The expression of b-FGF in our SHPT parathyroid tissue was significantly increased. These results are in line with prior observations [7] demonstrating the presence of b-FGF and its receptor in the PTG tissue of SHPT patients. The fact that the statistical difference remained even after reclassifying the specimens into b-FGF positive or negative suggests that this growth factor could be more relevant than VEGF-A in the SHPT hyperplastic glands angiogenic response. Certainly more studies are necessary to confirm these data.

In conclusion, our study showed that the hyperplastic parathyroid tissue from chronic kidney disease patients with secondary hyperparathyroidism presents an increased number of neovessels, accompanied with an increase in the angiogenic factors, VEGF-A and b-FGF. Studies using an animal model of secondary hyperparathyroidism could be very helpful to evaluate the reproducibility of these results and the possible role of angiogenesis inhibitors in the prevention and treatment of PTG hyperplasia associated with secondary hyperparathyroidism.

Acknowledgements. This work was supported by Grant 2537 (URDN) from Fundação para a Ciência e Tecnologia/FEDER. The authors wish to thank Ana Cristina Santos, MPH, from the Department of Hygiene and Epidemiology, School of Medicine, Porto University, for her assistance in the statistical analysis. The authors express their sincere appreciation to the Surgical and Pathology Departments of the Hospital S. João for providing the material used for this study.

Conflict of interest statement: João M. Frazão is a consultant and advisory board member for Amgen and Genzyme and served as an advisory board member for Abbott. The other authors have no conflict of interest to disclose.

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Received for publication: 29.5.07
Accepted in revised form: 26.2.08