Immunohistochemical investigation of angiogenic factors in parathyroid proliferative lesions

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Abstract

Objective: The pathological distinction between parathyroid neoplasms and hyperplasias remains difficult in several cases. Endoglin (CD105) is a proliferation-associated and hypoxia-inducible protein abundantly expressed in angiogenic endothelial cells. Vascular endothelial growth factor (VEGF) induces angiogenesis and VEGF-R2 is a tyrosine kinase receptor expressed early in development by endothelial cell precursors. We attempted to examine whether immunohistochemical expression of CD105, VEGF and VEGF-R2 may be useful in distinguishing between parathyroid hyperplasia and neoplasia as well as to elucidate, to some extent, the mechanism of neovascularization in proliferative lesions of the parathyroid gland.

Design: Tissue specimens were taken from 38 patients with primary hyperparathyroidism (HPT) (17 adenomas and 21 primary hyperplasias) and from 30 patients with secondary HPT. Normal glands served as controls.

Methods: In a standard immunohistochemical procedure, monoclonal antibodies to endoglin, VEGF and VEGF-R2 were applied to detect angiogenic endothelial cells. Immunostaining was estimated by image analysis and statistical analysis was subsequently performed.

Results: Positive CD105 immunoreaction was significantly increased in parathyroid adenomas by comparison with primary hyperplasias ($P=0.033$) and with secondary hyperplasias ($P=0.033$). When parathyroid adenomas, primary hyperplasia and secondary hyperplasia specimens were comparatively evaluated, VEGF immunoreaction was much more common in adenomas ($P=0.018$). In addition, in samples with secondary hyperplasia, VEGF-R2 immunoreactivity was positively linked with VEGF expression as well as with the apoptotic index of parathyroid cells ($P=0.038$ and $0.010$ respectively). In secondary hyperplasia specimens, an inverse correlation between cyclin D1 immunoeexpression and angiogenic indexes, such as CD105 and VEGF, was noticed ($P=0.007$ and $0.0017$ respectively).

Conclusions: This study shows increased angiogenesis in parathyroid adenomas compared with parathyroid proliferative lesions. In secondarily hyperplastic glands increased angiogenesis and increased apoptosis occur simultaneously; in the latter glands, the overexpression of cyclin D1 does not appear to be the genetic abnormality responsible for increased angiogenesis.

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Introduction

Hyperparathyroidism (HPT) is the most common disorder involving the parathyroid gland. The pathogenesis of primary and secondary HPT is almost completely different. Primary HPT refers to a syndrome caused by excessive secretion of parathyroid hormone by a parathyroid adenoma, primary hyperplasia of one or more parathyroids, or, in rare cases, parathyroid carcinoma (1). Parathyroid tumors are virtually always benign with a reported incidence of parathyroid carcinoma causing HPT in only 0.017% of cases (2). Secondary parathyroid hyperplasia is encountered principally in patients with chronic renal failure, although the disorder also occurs in association with vitamin D deficiency, intestinal malabsorption, Fanconi syndrome, and renal tubular acidosis (1); aberrant expression of several genes and growth factors is potentially involved in parathyroid tissue hyperplasia of secondary HPT (3). The pathological distinction between the different parathyroid lesions is, in many cases, problematic.

Angiogenesis is a multistep process, involving both the endothelium and the extracellular matrix. The angiogenic phenotype depends on the balance of proangiogenic growth factors and inhibitors, as well as interactions with the extracellular matrix, allowing for endothelial migration. Pro-angiogenic factors include vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGF-2), transforming growth...
factor (TGF-β1), placenta growth factor and angiopoietins. Inhibitors include cleaved products of other larger proteins that are not themselves inhibitors such as angiostatin and endostatin (4).

VEGF or VEGF-A belongs to the VEGF family which also includes VEGF-B, -C, -D and -E that via their receptors VEGFR-1, -2 and -3 are believed to participate in angiogenesis and lymphangiogenesis. VEGF, in particular, through its receptors VEGFR-1 and -2, induces angiogenesis, which is necessary for tumor growth, invasion and metastasis. Regulation of VEGF gene expression occurs via several different mechanisms, including hypoxia, growth factors and cytokines. Microvessel density (MVD) correlates with the concentration and expression of VEGF (4). VEGF-R2 is a tyrosine kinase receptor expressed early in development by endothelial cell precursors and is involved in angiogenesis.

Endoglin (CD105) is a receptor for TGF-β1 and TGF-β3, which modulates TGF-β functions via interaction with TGF-β receptors I and II and modifies the phosphorylation of downstream Smad proteins. It is expressed predominantly on the surface of endothelial cells as a 180 kDa homodimeric transmembrane glycoprotein. Other cell types including vascular smooth muscle cells, fibroblasts, macrophages, leukemic cells of pre-B and myelomonocytic origin, and erythroid precursors express CD105 at the transcriptional level. It is strongly expressed in blood vessels of tumor tissues (5).

We analyzed a series of non-familial parathyroid lesions to examine whether immunohistochemical expression of CD105, VEGF and VEGF-R2 may be useful in distinguishing between parathyroid hyperplasia and neoplasia as well as to elucidate, to some extent, the mechanism of neovascularization in proliferative lesions of the parathyroid gland, namely hyperplasias (primary or secondary) and adenomas.

Materials and methods

The specimens used for the study were 112 parathyroid glands from 68 hypercalcemic patients. The procedures followed were ethically approved. Parathyroid tissue was collected from 38 patients with primary HPT and from 30 patients with secondary HPT; the mean ± S.E.M. plasma total calcium levels were 3.04 ± 0.09 and 2.73 ± 0.06 mmol/l, respectively, and the plasma intact parathyroid hormone (PTH) levels were 140 ± 80 and 1199 ± 337 pg/ml respectively. Patient demographics are summarized in Table 1. All the secondary hyperparathyroid patients suffered from chronic renal failure. In each selected case, the diagnosis of the parathyroid lesion was definite. In detail, the adenoma diagnosis was based on enlargement of one gland, where the remaining glands were of normal size; a biopsy specimen from at least one of these glands was examined microscopically, revealing normal histology and was used as a normal control. Additionally, 10 parathyroid glands resected during thyroid surgery of nine normocalcemic patients were used as normal controls; seven of them were incidentally removed during thyroid surgery and the remaining three were located within the thyroid gland parenchyme. At least two glands were examined in each case of parathyroid hyperplasia. Of the 30 cases of secondary hyperplasia, 17 showed nodular growth and 13 showed diffuse growth. Both types contained both oxyphilic and chief cells in varying amounts. Primary hyperplasias were either diffuse (n = 15) or nodular (n = 6) and were composed predominantly of chief cells, although islands or nodules of oxyphilic were detectable in almost all cases.

All tissues were weighed (Table 1) and then routinely processed after a minimum of 4 h and a maximum of 24 h of fixation in buffered formalin. Prior to immunostaining, endogenous peroxidase activity was inhibited. Detection of the immunoreactive staining was carried out by the avidin–biotin–peroxidase complex method according to the manufacturer’s instruction (ABCComplex; Dako, Glostrup, Denmark) with diamobenzidine as chromogen. For endoglin (CD105), a mouse IgG1 monoclonal antibody (Clone SN6h; Diagnostic BioSystems, Pleasanton, CA, USA) was used at a dilution of 1:20 with 60 min incubation. Formalin-fixed paraffin-embedded tissue sections required predigestion with Protease XXXV. This antibody reacts with 95 kDa (monomer) and 190 kDa (dimer) proteins. CD105 binds to TGF-β1 and TGF-β3 with affinity but not to TGF-β2. For VEGF, a mouse IgG2b monoclonal antibody (Clone G153-694; BD Transduction Laboratories PharMingen, San Diego, CA, USA) was used at a dilution of 1:100 with 60 min incubation. The purified G153-694 is useful for detection of the immunoreactive staining was carried out by the avidin–biotin–peroxidase complex method according to the manufacturer’s instruction (ABCComplex; Dako, Glostrup, Denmark) with diamobenzidine as chromogen. For endoglin (CD105), a mouse IgG1 monoclonal antibody (Clone SN6h; Diagnostic BioSystems, Pleasanton, CA, USA) was used at a dilution of 1:20 with 60 min incubation. Formalin-fixed paraffin-embedded tissue sections required predigestion with Protease XXXV. This antibody reacts with 95 kDa (monomer) and 190 kDa (dimer) proteins. CD105 binds to TGF-β1 and TGF-β3 with affinity but not to TGF-β2. For VEGF, a mouse IgG2b monoclonal antibody (Clone G153-694; BD Transduction Laboratories PharMingen, San Diego, CA, USA) was used at a dilution of 1:100 with 60 min incubation. The purified G153-694 is useful for immunohistochemical staining. It has been used to

Table 1 Pathological cases and male (M) and female (F) patient demographics.

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<tr>
<th>Total no. of cases: 68</th>
<th>M:F = 1:2.2</th>
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<tr>
<td>Normal controls: 30</td>
<td>M:F = 1:1; mean age (years) 56.4; mean glandular weight (mg) 50.3±27.4</td>
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<tr>
<td>Adenomas: 17</td>
<td>M:F = 1:2.2; mean age (years) 57.4; mean glandular weight (mg) 1569±1683.2</td>
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<td>Hyperplasias: 51; primary: 21; secondary: 30</td>
<td>M:F = 1:2.4; mean age (years) 52.3; mean glandular weight (mg) 616.8±485.3; mean age (years) 51.7; mean age (years), 53.8</td>
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immunoprecipitate native human VEGF and to identify three isoforms of VEGF (165, 189, 206 aa) by Western blotting. For VEGF-R2, a mouse IgG1 monoclonal antibody (Clone 89 115; R&D Systems, Minneapolis, USA) was used at the same dilution as VEGF antibody. This antibody recognizes human VEGF-R2, which is a tyrosine kinase receptor expressed early in development by endothelial cell precursors and is involved in angiogenesis. For all three immunomarkers, tissue sections from an angiosarcoma served as positive controls. As a substitute ‘negative’ control, omission of the primary antibody was performed in each experiment and the respective slides remained totally unstained. Staining results were evaluated both quantitatively and semi-quantitatively. Only moderately to strongly immunoreactive cells were considered as positive; in detail, chief cells were considered immunopositive when a moderate or strong immunoreaction pattern was observed; as far as oxyphilic cells are concerned, only a strong intensity pattern was considered positive since this category of cells may give false positive results. First, the percentage of immunopositive cells among all cells examined was evaluated in each case for all three markers. Then the cases were categorized as negative and positive; a cut-off point of 10% was used.

All specimens of the present study had been previously evaluated with regard to various immunomarkers (i.e. Ki-67, cyclin D1, Fhit protein) and apoptotic index of parathyroid cells. The detection of apoptotic cells in parathyroid tissues had been performed with an antibody specifically reactive with single stranded DNA; in detail, this antibody reacts specifically with deoxycytidine and requires stretches of ssDNA of at least 25–30 bases in length for the binding (6). Importantly, in contrast to the TUNEL method (7–9), monoclonal antibodies to ssDNA are considered specific for apoptotic cell death and do not detect necrotic cells. For cyclin D1, the H-295 rabbit polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), raised against a recombinant protein corresponding to amino acids 1–295 representing full-length cyclin D1 of human origin, was used at a dilution of 1:150 with overnight incubation. The respective information was drawn from our archival data base (10).

**Results**

A minimal expression of the three immunomarkers was detectable in the normal parathyroid tissues examined; there was no difference in the pattern of immunostaining for any of the three markers evaluated between the group of normal parathyroids from normocalcemic patients and that of normal parathyroids from hypercalcemic patients. In immunopositive samples, the percentage of positive cells ranged from 15% to 63%, 12% to 46%, and 11% to 21% for CD105, VEGF and VEGF-R2 respectively. VEGF immunostaining was cytoplasmic and, apart from parathyroid cells, VEGF was expressed in stromal fibroblasts and rarely in endothelial cells. VEGF-R2 immunoreactivity was characterized by a membranous/cytoplasmic staining pattern and, apart from parathyroid parenchymal cells, was noticed in several endothelial cells.

As far as CD105 immunostaining is concerned, positive CD105 immunoreaction was detected in 49 (66.2%) of all samples examined. The presence of CD105-positive vessels was significantly increased in parathyroid adenomas (Fig. 1A) by comparison with all normal controls ($P=0.003$), with primary hyperplasias ($P=0.033$) and with secondary hyperplasias ($P=0.033$). In addition, in secondary hyperplasia specimens, CD105 and cyclin D1 immunoreactivity were inversely correlated to each other ($P=0.007$) (Fig. 2A). No association emerged between CD105 immunopositivity and Ki67 index.

With regard to VEGF immunostaining, VEGF was positively expressed in 49 (66.2%) of all samples examined. Positive VEGF immunoreaction was more frequently detected in parathyroid adenomas (Fig. 1C) in comparison with all normal controls ($P < 0.001$) and in primary hyperplasias compared with all normal parathyroid glands ($P=0.006$). Moreover, secondary hyperplasia specimens and all normal controls were found to differ, although with a borderline statistical significance ($P=0.061$) in regard to the incidence of VEGF immunopositivity. When parathyroid adenomas, primary hyperplasia and secondary hyperplasia specimens were evaluated comparatively, VEGF immunoreactivity was much more common in adenomas ($P=0.018$). When all primary lesions were taken together as a group, a statistically significant difference was detected by comparison with secondary lesions ($P=0.019$); in other words, VEGF, in primary lesions, was expressed more frequently than in secondary hyperplasia samples. Interestingly, in the latter samples, an inverse correlation between VEGF and cyclin D1 immunostaining was noticed ($P=0.0017$) (Fig. 2B).

As far as VEGF-R2 is concerned, it was positively expressed in 33 (44%) of all samples examined (Fig. 1E). In all pathological parathyroid samples VEGF-R2 was more commonly expressed compared with all normal controls ($P=0.005, 0.017$ and $0.009$ for primary hyperplasias, parathyroid adenomas and secondary

**Statistical analysis**

The chi-squared statistic was employed to investigate the association between patient groups and CD105 or VEGF. All tests were two-sided and the level of statistical significance was set at 5%. In addition, non-parametric statistics (Kruskal–Wallis ANOVA or Mann–Whitney U-test) were performed to investigate the association between patient groups and VEGF-R2. All tests were two-sided and the level of statistical significance was set at 5%.
hyperplasias respectively) (Fig. 3). No difference emerged when the three groups of pathological samples were compared with each other ($P = 0.871$). In addition, in samples with secondary hyperplasia, VEGF-R2 immunoreactivity was positively linked with VEGF expression as well as with the apoptotic index of parathyroid cells ($P = 0.038$ and $0.010$ respectively).

The group of parathyroid tumors with ‘negative’ immunoexpression (less than 10% of cells stained) did not differ from the group of immunopositive tumors at a statistically significant level with regard to proliferative index or serum calcium and PTH levels.

**Discussion**

Elevated levels of CD105 expression have been detected on vascular endothelial cells in tissues undergoing active angiogenesis, such as regenerating and inflamed tissues or tumors. In particular, endoglin is largely expressed in small and likely immature tumor vessels, as demonstrated in breast, prostate and gastric cancer (11). Vascular density has been assessed by counting vessels labeled using immunohistochemistry with antibodies to different endothelial markers on both frozen and paraffin-embedded sections. Most commonly used antibodies are directed against the endothelial antigens factor eight-related antigen (F8), CD34, CD31 and UEA1 (4). Pan-endothelial markers, such as anti-CD34 antibody, stain all blood endothelial cells, including mature and immature cells, and do not allow the distinction between newly formed vessels, responsible for angiogenesis, and preexisting vessels (12). Immunohistochemistry studies have revealed that anti-CD105 antibodies show a greater specificity for tumor vasculature compared with pan-endothelial markers.

Our study shows that CD105 endothelial expression is significantly higher in parathyroid adenomas compared with normal glands and parathyroid proliferative lesions, such as primary and secondary hyperplasias. Moreover, our data are consistent with the fact that benign proliferative lesions have higher MVD than normal tissue. The finding of a higher vascularization in adenomas compared with primary hyperplasias might be related to the degree of cellular proliferation of the glands, as several studies have revealed higher cellular proliferation indexes in the former than in the latter (13, 14). However, in the samples of the present study, no differences had emerged between neoplastic and hyperplastic parathyroid lesions, at least with respect to Ki67 immunopositivity (10). Therefore, the difference with regard to CD105 immunoreactivity between adenomas and primary hyperplasias is not likely to be justified by any difference in their respective proliferative indexes. Additionally, the difference in positive CD105 immunoreaction fits with
the clinical observation that primary hyperplasia is a distinct clinical entity from parathyroid adenoma and may thus provide a useful tool for the histological distinction between them (12). CD105 was expressed exclusively in endothelial cells in both tumoral glands and to a significantly lesser degree in normal parathyroids. The distribution of VEGF and VEGF-R2 immunoreactivity concerned mainly parathyroid parenchymal cells and chief cells, in particular, both in tumoral glands and in normal controls, although with a minimal expression in the latter. Oxyphilic cells were occasionally immunopositive, and they were calculated in the percentage of immunopositive cells only when they were intensely stained.

The present study demonstrates that VEGF expression is significantly higher in parathyroid adenomas compared with normal controls, and primary and secondary hyperplasias. Additionally, VEGF positive immunoreaction was detected more frequently in primary hyperplasias than in normal controls and also in primary lesions in comparison with secondary hyperplasias. The latter were found to differ from normal tissues, although with a borderline statistical significance, with regard to the incidence of VEGF immunopositivity. Studies in other tissues (i.e. in colorectal cancer) have shown that the expression of VEGF is positively related to MVD, further reinforcing the interrelation of VEGF and MVD (15). In particular, CD105-MVD was significant and more closely correlated with VEGF score than CD34-MVD in non-small-cell lung cancer (16). However, in parathyroid tissue no significant association between VEGF expression and CD105 immunostaining emerged. The lack of correlation between CD105 and VEGF expression suggests that VEGF, on its own, is not likely to be the primary pro-angiogenic factor in parathyroid tissue. Another pro-angiogenic factor that appears to be involved in angiogenesis in parathyroid tissue is FGF-2. It is an important factor that regulates angiogenesis in parathyroid tumors. It has a synergistic effect on VEGF-A in cultured endothelial cells and has been shown to be expressed in parathyroid adenomas and secondary hyperplasias (12).

As far as VEGF-R2 is concerned, its expression is higher in parathyroid adenomas and proliferative lesions than in normal controls. It is noteworthy that any differences observed in the angiogenic profile between parathyroid tumors did not exert any influence in the clinical, biochemical, proliferative and apoptotic characteristics of the tumors. No difference emerged either when adenomas and hyperplastic parathyroid tissues were compared with each other.

As far as VEGF-R2 is concerned, its expression is higher in parathyroid adenomas and proliferative lesions than in normal controls. It is noteworthy that any differences observed in the angiogenic profile between parathyroid tumors did not exert any influence in the clinical, biochemical, proliferative and apoptotic characteristics of the tumors. No difference emerged either when adenomas and hyperplastic parathyroid tissues were compared with each other.

It is of interest that in secondarily hyperplastic glands VEGF-R2 immunoreactivity was positively correlated with VEGF expression as well as with the apoptotic index of parathyroid cells. The positive correlation seen between VEGF expression and VEGF-R2, particularly in secondary hyperplasia specimens, may suggest that both have an important influence on the course of
secondary hyperplasias. However, a similar inter-
relation in serum has been reported in patients with
chronic lymphocytic leukemia (17). With regard to the
correlation between VEGF-R2 and the apoptotic index,
it is known that secondary hyperplasia is associated
with an increase in apoptosis. It is of interest that the
uremic state appears to stimulate apoptosis in other cell
types apart from parathyroid cells (3). Both systemic
and local factors contribute to an acceleration of
programmed cell death in parathyroid tissue, such as
disturbed vitamin D metabolism and increased cyto-
plasmic calcium concentration (9). In secondarily
hyperplastic cases, the enhancement of parathyroid
tissue apoptosis appears to compensate for the increase
in parathyroid cell proliferation also detected in these
lesions. However, because parathyroid gland mass
grows progressively with time, the increase in apoptosis
is probably insufficient to counterbalance enhanced
proliferation in the long run (10). The correlation seen
between VEGF-R2 and apoptotic index suggests that
increased angiogenesis, increased apoptosis and dereg-
ulated proliferation occur simultaneously during the
progression of secondary hyperplasia.

Parathyroid adenoma 1 (PRAD1) oncogene belongs
to the cyclin D1 family (cyclin D1) and thus may affect
cell growth directly. D cyclins regulate the G1 phase
of the cell cycle by inducing the phosphorylation of the
retinoblastoma (Rb) tumor-suppressor protein, which
leads to inactivation of Rb and promotes cell replication.
Thus, deregulation or overexpression of cyclin D1 in a
parathyroid cell could accelerate progression from the
G1 into the S phase, thereby causing excessive cell
proliferation without necessarily inducing a malignant
phenotype (18). In secondarily hyperplastic specimens,
angiogenic factors were quite highly expressed, while
levels of cyclin D1 were rather low (6). With regard to
the inverse correlation between cyclin D1 immunoe-
expression and angiogenic indexes, such as CD105 and
VEGF, in secondary hyperplasia specimens, it is of
interest that in secondarily hyperplastic glands, over-
expression of cyclin D1 does not seem to be the exclusive
genetic abnormality responsible for monoclonal proliferation of parathyroid
cells and increased angiogenesis.

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