

Comparative study of the expression of proteins involved in the cell cycle in renal secondary hyperparathyroidism

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Background. In renal hyperparathyroidism, parathyroid cell proliferation seems to play a key role in the progression of the disease. Therefore, G1/S transition, a main cell cycle regulatory step, could be deregulated in these patients.

Methods. One hundred and one parathyroid glands, taken from parathyroidectomies performed on 41 patients on hemodialysis (HD), and 15 glands, taken from 7 patients with post-transplantation persistent hyperparathyroidism (HPT), were studied. Twelve normal parathyroid (PT) glands were used as the control. Biochemical data, immunohistochemical (IHC) profiles of G1/S transition regulators belonging to the two main pathways (cyclin D1/p16^{INK4A}/pRb and p14^{ARF}/p53/MDM2), and proliferation rate (Ki67) were correlated.

Results. All of the other proteins differed from normal IHC profiles in both groups that showed significant higher proliferating rates, decreases in p27^{KIP1}, pRb, and cyclin D1, as well as increases in p16^{INK4A}, p53, MDM2, and p21^{WAF1} levels, in comparison with normal PT glands, with the exception of cyclin D3. Contrary to patients with HPT who were on hemodialysis, in post-transplantation HPT, consistent correlations between biochemical data and IHC profiles were obtained.

Conclusion. In both groups IHC profiles of proteins involved in G1/S transition regulation significantly differed from normal PT glands. The results support partial reversion to normal IHC profile in post-transplantation HPT.

Renal secondary hyperparathyroidism (HPT) is a very frequent condition that arises in hemodialysis (HD) patients and ultimately requires parathyroidectomy in a substantial proportion of cases [1]. Its clinical course and pathologic evolution have been reasonably well defined, but its pathogenesis remains incompletely defined [2–4]. On the other hand, persistent post-transplantation HPT occurs in a subgroup of patients with functioning renal

grafts, some cases requiring parathyroidectomy [5, 6]. The cause of this is unknown, although it is reasonable to hypothesize that persistence of remnant PT cell populations harbor mutations, rendering them at least partially refractory to physiologic homeostatic signals.

In both conditions, parathyroid (PT) cell growth regulation seems to play an important role in the progression of the disease. Currently, there is an important gap in the knowledge linking classic PT physiology with recent advances in our understanding of cell cycle regulation at the molecular level [3, 4, 7–9]. For these reasons we considered it relevant to address mechanisms involved in PT cell cycle regulation under these circumstances. Until recently, they remain basically unexplored, especially in the case of post-transplantation HPT.

G1/S transition probably constitutes the main cell cycle regulatory step in the majority of cell types, either reactive or after neoplastic transformation [10]. G1/S transition regulators belong to two main interconnected pathways: cyclin D1/p16^{INK4A}/pRb and p14^{ARF}/p53/MDM2 [11, 12]. We, thus, hypothesized that G1/S transition regulation could be altered in both pathways. Based on this premise, and taking into account our own studies on PT adenomas [13, 14], we set up to systematically explore immunohistochemical (IHC) profiles of several key proteins belonging to the mentioned pathways.

METHODS

Patients and samples

For this study we selected 101 PT glands (30 with diffuse and 71 with nodular hyperplasia) from 41 patients with HPT on hemodialysis (21 males, 20 females, mean age 46.7 ± 18.5 years; mean time on hemodialysis: 74.0 ± 40.11 months) and 15 PT glands from 7 patients with post-transplantation HPT (1 male, 6 females; mean age 51.9 ± 12.3 years; post-transplantation time: 66.5 ± 36.5

Key words: hyperparathyroidism, G1/S, regulation, protein, immunohistochemistry, expression.

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Table 1. G1/S transition: HDHPT versus normal PT

	HDHPT	Normal PT	P value
pRb	17.3 ± 0.8	32.7 ± 4.2	<0.05
Cyclin D1	21.1 ± 0.7	29.5 ± 2.1	<0.05
Cyclin D3	2.3 ± 0.2	2.7 ± 0.9	NS
p16 ^{INK4}	4.1 ± 0.3	0	<0.05
p27 ^{KIP1}	32.1 ± 0.9	55.8 ± 4.4	<0.05
p53	4.8 ± 0.3	0 ± 1.2	<0.05
MDM2	10.0 ± 0.52	2.1 ± 2.7	<0.05
p21 ^{WAF1}	5.0 ± 0.3	0.9 ± 1.6	<0.05

All values refer to percentage of positive cells and are shown as mean ± SEM. Abbreviations are: HDHPT, hemodialyzed patients with hyperparathyroidism; PT, parathyroid; NS, not significant. $P < 0.05$ is statistically significant. P is not significant at >0.05 .

months), in which total parathyroidectomy and PT autograft had been performed. The glands were aleatorily selected from among the largest from each patient. Twelve normal PT glands, from 6 males and 6 females, removed in the course of unrelated pathologies, were used as control. In all the cases, basic laboratory biochemical parameters corresponding to the presurgical period (calcium, phosphorus, alkaline phosphatase, and parathyroid hormone [PTH]) were obtained.

Immunohistochemistry

After conventional processing, IHC stainings with monoclonal (Mc) Abs against Ki67 (proliferating rate, PR), cyclins D1 and D3, pRb, p16^{INK4}, p53, MDM2, p21^{WAF1} and p27^{KIP1}, were performed in paraffin sections as previously described [13, 14], with the exception of cyclin D3. Cyclin D3 was detected with Mc Ab DCS-22 from Novocastra (Newcastle, UK), at 1:50 dilution. Labeling Index (LI) was measured with a CAS200 BV Cytometry Digital Image System (Becton Dickinson, Leiden, The Netherlands), and was expressed as the percentage of positive cells. At least 1000 cells in PT glands with diffuse hyperplasia and 1000 cells in each nodule in PT glands with nodular hyperplasia were counted.

Statistical analysis

All the data were expressed as mean ± SEM. Statistical analysis was carried out with NCSS 2000 statistical package (Number Cruncher Statistical Systems, Kaysville, UT, USA). Correlation coefficients were calculated using Pearson and Spearman correlations. Analysis of variance (ANOVA) was performed by Scheffe or Kruskal-Wallis multiple comparison tests when values followed a normal or non-normal distribution, respectively. Significance level was always $P < 0.05$.

RESULTS

Biochemical data correlate with IHC profiles in post-transplantation HPT but not in HD patients with HPT

No correlations were found between biochemical data and hemodialysis time or IHC profiles in HD patients

Table 2. G1/S transition: Post-transplantation HPT versus normal PT

	Post-transplantation HPT	Normal PT	P value
pRb	12.3 ± 2.6	32.7 ± 4.2	<0.05
Cyclin D1	8.6 ± 2.4	29.5 ± 2.1	<0.05
Cyclin D3	2.3 ± 0.7	2.7 ± 0.9	NS
p16 ^{INK4}	4.9 ± 1.1	0	<0.05
p27 ^{KIP1}	44.6 ± 2.7	55.8 ± 4.4	NS
p53	4.1 ± 0.9	0 ± 1.2	<0.05
MDM2	16.7 ± 1.6	2.1 ± 2.7	<0.05
p21 ^{WAF1}	4.2 ± 1.2	0.9 ± 1.6	<0.05

All values refer to percentage of positive cells and are shown as mean ± SEM. Abbreviations are: HPT, hyperparathyroidism; PT, parathyroid; NS, not significant. $P < 0.05$ is statistically significant. P is not significant at >0.05 .

with HPT. In contrast, in post-transplantation HPT, proliferation rates (PR) ($r = -0.79$), cyclins D1 ($r = -0.98$), D3 ($r = -0.99$), and p16^{INK4A} ($r = -0.66$) negatively correlated with serum phosphorus and positively with serum calcium levels ($r = 0.86, 0.99, 0.99$, and 0.74 , respectively), while PTH serum levels correlated positively with PR ($r = 0.70$) and p16^{INK4A} ($r = 0.83$) and negatively with pRb ($r = -0.99$), p27^{KIP1} ($r = -0.99$), and all p53 pathway members (p53: $r = -0.89$; MDM2: $r = -0.83$; p21^{WAF1}: $r = -0.86$).

IHC profiles of HD patients with HPT and post-transplantation HPT differ from normal PT tissue

PR: In normal PT glands, the PR (0.18 ± 0.09) was significantly lower than that obtained in both groups (Table 1). In PT glands from HD patients with HPT, PR (3.60 ± 0.24) was significantly higher compared with post-transplantation HPT (1.25 ± 0.74). In this last group, PR correlated negatively with p27^{KIP1} ($r = -0.77$) and pRb ($r = -0.78$) and positively with cyclins D1 ($r = 0.89$) and D3 ($r = 0.84$) and p16^{INK4A} ($r = 0.98$).

With the exception of cyclin D3 and partly p27^{KIP1}, all the other proteins differed from normal PT gland IHC profiles in both groups.

Cyclin D1/p16^{INK4A}/pRb pathway: Both groups showed significant decreases in pRb and cyclin D1, as well as increases in p16^{INK4A} levels, in comparison with normal PT glands. It was especially remarkable for cyclin D1. Cyclin D1 levels were significantly lower in post-transplantation HPT than those in HD patients with HPT (Table 2). Cyclin D3 did not show significant deviations from normal PT glands (Table 3).

p14^{ARF}/p53/MDM2 pathway: Both groups showed similar results, with a significant downregulation of p27^{KIP1}, while p53, MDM2, and p21^{WAF1} were clearly upregulated. p27^{KIP1} levels present in post-transplantation HPT, although clearly lower in comparison with normal PT glands, did not achieve statistical significance.

DISCUSSION

In renal hyperparathyroidism, PT cell hyperplasia, either associated with mono- or multiclonal growth, arises

Table 3. G1/S transition: HDHPT versus post-transplantation HPT

	HDHPT	Post-transplantation HPT	P
pRb	17.3 ± 0.8	12.3 ± 2.6	NS
Cyclin D1	21.1 ± 0.7	8.6 ± 2.4	<0.05
Cyclin D3	2.3 ± 0.2	2.3 ± 0.7	NS
p16 ^{INK4}	4.1 ± 0.3	4.9 ± 1.1	NS
p27 ^{KIP1}	32.1 ± 0.9	44.6 ± 2.7	<0.05
p53	4.8 ± 0.3	4.1 ± 0.9	NS
MDM2	10.0 ± 0.52	16.7 ± 1.6	NS
p21 ^{WAF1}	5.0 ± 0.3	4.2 ± 1.2	NS

All values refer to percentage of positive cells and are shown as mean ± SEM. Abbreviations are: HDHPT, hemodialyzed patients with hyperparathyroidism; HPT, hyperparathyroidism; NS, not significant. $P < 0.05$ is statistically significant. P is not significant at >0.05 .

as a result of yet unknown pathogenic events. Until recently, several genetic loci have been explored unsuccessfully [2, 3, 15]. Based on comparative genomic hybridization findings, potential roles for other yet uncharacterized loci have been recently suggested [4]. The development of cell and tissue hyperplasia obviously has to be placed in close relation with cell cycle regulation. Currently, there is very limited available information concerning cell cycle regulation in PT hyperplasia. In this study, we have systematically explored the IHC expression of several key cell cycle proteins in an attempt to demonstrate G1/S transition deregulation in HD patients with HPT and post-transplantation HPT.

As a reflection of hyperplasia, PR in PT glands is not greatly increased. The majority of the studies in PT pathology address only a limited set of cell cycle regulatory proteins. Most of them study PR. They report on PR increases in PT glands in humans [9, 16–20] and in rats [21], in accordance with our own data. In our series, the PR found in HD patients with HPT was higher than in those with post-transplantation HPT, and similar to that reported previously by us in PT adenomas [14]. It correlates with the different clinical background and macroscopic anatomic findings (data not shown).

Our results in the pRb pathway in HD patients with HPT are in accordance with increased PR. Cyclin D1 downregulation is a striking finding, but it should be interpreted cautiously in the context of high physiologic expression in normal PT glands. The data previously reported are discordant because some authors have found cyclin D1 overexpression [9, 22], whereas others have not [23]. To reconcile these findings, it should be recalled that normal PT glands included as controls in some studies come from patients with PT adenomas, in which the tumor expresses high cyclin D1 levels and the remnant PT glands probably showing skewed IHC profiles. p27^{KIP1} downregulation has been already reported by others [20] and supports high PR found in these patients. These same authors showed p27^{KIP1} downregulation in PT adenomas and carcinomas, lending fur-

ther support to the potential pathogenic role of p27^{KIP1} in PT proliferative lesions.

In comparison with HD patients with HPT, post-transplantation HPT patients showed lower cyclin D1 and higher p27^{KIP1} levels. In both groups, progressive pRb inactivation and p27^{KIP1} downregulation could play a main role, allowing PT cell proliferation [20].

p16^{INK4A}, p53, and p21^{WAF1} upregulation could also be envisioned, based on the absence of recently reported mutations, as attempts to slow down cell proliferation and/or induce apoptosis and cell senescence as has been proposed previously [12]. p53 overexpression could be functional [24] and related to MDM2 and p21^{WAF1} upregulation found in both groups [25, 26]. These are rather unusual findings, taking in account the benign nature of the disease. They are similar to those previously reported by us in PT adenomas [13]. In PT adenomas, at least, we have discarded the presence of MDM2 genomic amplification and suggested the existence of post-transcriptional regulatory mechanisms (data from the International Conference on Basic and Clinical Aspects of Cell Cycle Control, Siena, Italy, 2000).

The absence of strong biochemical and IHC correlations in HD patients with HPT in our study probably underlie clinical and tissue heterogeneity. In contrast, we found significant positive correlations between PR, both cyclins, and p16^{INK4A}, with calcium levels and negative correlations with serum phosphorus levels in patients with post-transplantation HPT. In this context it must be remembered that in our study, patients with post-transplantation HPT had consistently higher calcium levels (data not shown) and lower cyclin D1 levels compared to HD patients with HPT. This is in accordance with experimental data reporting cyclin D1 downregulation in response to increased calcium levels [27]. On the other hand, negative correlations between PR and phosphorus levels in post-transplantation HPT probably place this group in a similar situation to primary hyperparathyroidism and opposite of that found in HD patients with HPT, in which, at least at the beginning, phosphorus retention appears to be a key factor in cell proliferation [15, 28]. All of these findings are also in accordance with PTH correlations in post-transplantation HPT.

In conclusion, our data confirm the presence of significant differences in IHC profiles of proteins involved in G1/S transition regulation in both groups (HD patients with HPT and post-transplantation HPT) in comparison with normal PT glands. They also suggest a “partial reversion” to normal G1/S transition regulation in post-transplantation HPT, based on the presence of lower PR and relatively homogeneous IHC and biochemical profiles compared to HD patients with HPT. All these data should be reinterpreted in the future with a more precise knowledge of the pathogenic mechanisms involved in renal HPT.

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