Abstract

Objective: The pathological distinction between parathyroid neoplasms and hyperplasias remains difficult. Changes in cell cycle control may lead to clonal proliferation and precede tumorigenesis. The parathyroid adenoma 1 oncogene, subsequently identified as the gene encoding cyclin D1, has been shown to be important to parathyroid tumour development. In addition to cell proliferation, the mechanisms of parathyroid cell turnover include apoptosis. The tumour-suppressor activity of the fragile histidine triad gene (FHIT) is linked to its proapoptotic function and cell cycle control. We attempted to evaluate the cellular proliferative kinetics and apoptotic function of the parathyroid glands in patients with non-familial hyperparathyroidism (HPT).

Design: Tissue specimens were taken from 40 patients with primary HPT (17 adenomas, two carcinomas 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 Ki-67 antigen and single-stranded DNA were applied to detect cycling and apoptotic cells respectively; polyclonal antibodies to cyclin D1 and Fhit protein were used. Immunostaining was estimated by image analysis and statistical analysis was subsequently performed.

Results: Significantly higher proliferative and apoptotic indexes were detected in the diseased glands in comparison with normal controls. In neoplastic and secondarily hyperplastic glands, apoptotic indexes were higher than in primarily hyperplastic glands; the difference between neoplastic and primarily hyperplastic glands was statistically significant ($P = 0.034$). Cyclin D1 was overexpressed in a considerable proportion of tumours (68.4%). A reduction of Fhit protein immunoreactivity was selectively noticed in carcinomas.

Conclusions: In primary hyperplasia, the remarkable proliferation of parathyroid glands may be due to the reduction of the apoptotic process. FHIT gene abnormalities are worthy of investigation in parathyroid carcinogenesis.
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) tumour-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 (6).

The fragile histidine triad gene (FHIT) is located at chromosome region 3p14.2 (7); its inactivation is being reported in a large fraction of human cancers. The Fhit protein has been proposed to act as a tumour suppressor in lung carcinoma cells by sending a proapoptotic signal and suppressing tumorigenesis of transfected cells (8). There is accumulating evidence that the tumour-suppressor gene FHT is involved in the regulation of apoptosis and in cell cycle control (9).

We analysed a series of non-familial parathyroid lesions to examine whether immunohistochemical expression of the above cell cycle regulators, apoptotic rate and Fhit protein immunoreactivity may be useful in distinguishing between parathyroid hyperplasia and neoplasia as well as to elucidate, to some extent, the mechanisms of proliferative lesions of the parathyroid gland, namely hyperplasias (primary or secondary), adenomas and carcinomas.

**Materials and methods**

The specimens used for the study were 112 parathyroid glands from 70 hypercalcemic patients. The procedures followed were ethically approved. Parathyroid tissue was collected from 40 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 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. The diagnosis of carcinoma was based on metastatic spread in cervical nodes. 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. An additional ten parathyroid glands resected during thyroid surgery of nine normocalcaemic patients were used as normal controls. 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 oxyphils 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 diaminobenzidine as chromogen. For Ki-67, a mouse IgG 1 monoclonal antibody (Clone MB67; Neo Markers, Fremont, CA, USA) was used at a dilution of 1:150 with overnight incubation; for cyclin D1, the H-295 rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used at a dilution of 1:1:200, overnight. Heat-induced epitope retrieval was performed by microwaving in a 1480 W oven for all antibodies. Permeablization in saponin, pronase E treatment, and heating in formamide were necessary for F7-26 immunostaining (according to the Chemicon detection protocol). Substitute negative controls consisted of omission of primary antibody and incubation with preimmune serum instead. Human intestinal mucosa was used as a positive control for Ki-67; Fhit expression in normal cells served as positive control. Data on immunostaining results for int-2 gene product were available for all samples from a previous study (10).

Image analysis was performed for the objective quantification of staining results for all immuno-
markers except for Fhit protein which was generally overexpressed. Only strongly immunoreactive nuclei were counted as positive. As cells in diffuse and nodular hyperplasias – including single nodules – show different characters, in cases with nodular and diffuse areas, these were separately evaluated; in addition, their mean value was taken into account (the latter is shown in Table 2). Images were acquired using a Zeiss Axioslab microscope (Carl Zeiss GmbH, Jena, Germany) with a mechanical stage, fitted with a SONY-iris CCD videocamera (SONY Corp., Tokyo, Japan); the latter was connected to a Pentium II personal computer located with the appropriate image analysis software. Slides were examined at high power magnification (× 400). A first elaboration of digital images was realized by using the Image Scan Pro Software (Science GmbH, Erkrath, Germany). Further evaluation took place via the Colour Estimator version 2.0, a specific application developed in the Department of Pathology of Athens University in a Microsoft Visual Basic 5.0 (Microsoft Corp., Redmond, WA, USA) environment for the evaluation of histochemical and immunohistochemical colour images. More specifically, we calculated the ratio expressed in percentages between the number of immunohistochemically positive nuclei and at least 500 nuclei (stained and unstained) after examining at least two sections for each sample; these values are given in Table 2. The sample of 500 cells per case was chosen on the basis of a summation average graph.

**Statistical analysis**

Initial analysis involved transformation of ssDNA and Ki-67 values into natural logarithms because of deviation from data normality. The distribution of cyclin D1 data did not exhibit normality properties after any transformation. Therefore, upparametric statistics were employed for the analysis of this specific variable. Statistical analysis for parametric data (ssDNA, Ki-67 indexes), included one-way analysis of variance followed by Dunnett’s test as post hoc test for comparison between the group of normal controls and each other group of interest as well as between nodular and diffuse areas in pathological samples. As mentioned before, cyclin D1 was analysed by aparmetric statistics (i.e. Kruskal–Wallis analysis of variance with ranks and chi-square for the comparison between the control group and each of the other groups). Statistical significance was set at 5%.

**Results**

Ki-67-immunopositive cells as well as apoptotic cells were detectable in all specimens (Figs 1 and 2). Cyclin D1 was overexpressed (in more than 20% of parathyroid cells; Fig. 3) in 68.4% of neoplasms (in 11/17 adenomas and in the two carcinomas), but it could be detected, at lower percentages of parathyroid cells, in 66.6% of cases with primary hyperplasia and in 60% of cases with secondary hyperplasia. The threshold of 20% for overexpression of cyclin D1 protein has been used previously (11). All normal controls were practically cyclin D1 immunonegative (immunopositivity scores <1%). Table 2 demonstrates the mean immunopositivity scores for Ki-67, cyclin D1 and ssDNA in the sample groups examined. The above three immunomarkers demonstrated a nuclear staining pattern and were detectable in both nodular and diffuse areas of the pathological samples, irrespective of the sizes of the glands, weight and any other clinical parameter assessed. No statistically significant differences emerged between diffuse and nodular areas in hyperplasias in terms of proliferation. In glands of increased size, a comparative reduction in apoptotic index was occasionally noticed, but the size of our sample did not permit

| **Table 2** The percentages of immunoexpression of proliferation markers and apoptosis in the specimen groups examined. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| **Ki-67 index** | **Mean value**  | **Standard error of the mean** | **Minimum** | **Maximum** |
| Normal controls (n = 10) | 0.19 | 0.06 | 0.02 | 0.50 |
| Neoplasms (n = 19) | 2.84 | 0.21 | 0.10 | 15 |
| Primary hyperplasia (n = 21) | 3.38 | 0.19 | 0.20 | 20 |
| Secondary hyperplasia (n = 30) | 3.14 | 0.18 | 0.10 | 20 |
| **Cyclin D1 index (in immunopositive cases)** | **Mean value**  | **Standard error of the mean** | **Minimum** | **Maximum** |
| Neoplasms (n = 13) | 27.353 | 6.919 | 0.20 | 95 |
| Primary hyperplasia (n = 14) | 14.543 | 4.995 | 0.01 | 60 |
| Secondary hyperplasia (n = 18) | 3.690 | 1.087 | 0.01 | 24 |
| **Apoptotic index** | **Mean value**  | **Standard error of the mean** | **Minimum** | **Maximum** |
| Normal controls (n = 10) | 0.14 | 0.08 | 0.01 | 0.80 |
| Neoplasms (n = 19) | 6.66 | 0.44 | 0.20 | 23.00 |
| Primary hyperplasia (n = 21) | 3.97 | 0.37 | 0.10 | 17.00 |
| Secondary hyperplasia (n = 30) | 5.84 | 0.34 | 0.10 | 24.00 |
statistical comparisons within each group of diseased glands. There appeared to be no difference between oxyphilic cells and chief cells in terms of stainability or the number of positive cells. Actually, chief cells predominated in all adenomas examined as well as in the remnants of ‘normal’ tissue outside the adenoma capsule. Cellular proliferative activity, as evidenced by Ki-67 immunostaining, appeared to be low in the normal rim of the adenomas as well as in normal controls; the latter demonstrated significantly lower Ki-67 and apoptotic indexes by comparison with each pathological group (i.e. neoplasms ($P = 0.004$ and $P = 0.001$ for Ki-67 and apoptotic index respectively), primary hyperplasia ($P = 0.001$ and $P = 0.051$) and secondary hyperplasia ($P = 0.001$ and $P = 0.001$)).

No significant differences were detectable among pathological groups with regard to Ki-67 or cyclin D1 immunoreactivity rates. However, as far as the apoptotic index is concerned, higher values were detected in neoplasms and cases of secondary hyperplasia than in cases of primary hyperplasia (Table 2); of the above-mentioned differences, the one between the group of neoplasms and the group of primary hyperplasia reached statistical significance ($P = 0.034$). In other words, significantly lower apoptotic indexes were noticed in the cases of primary hyperplasia compared with adenomas and carcinomas.

Fhit protein was constantly expressed with a cytoplasmic granular staining pattern in parathyroid cells of normal controls and all pathological samples except for the two carcinomas; in the latter, a reduction of Fhit protein was noticeable in neoplastic cells (Fig. 4). Finally, no correlation was noted between...
int-2 immunostaining and any of the markers examined in these specimens.

Discussion

The precise mechanisms involved in the control of parathyroid cell growth, division and death, and hence in the regulation of parathyroid size (10), are not known at present. Parathyroid adenomas are suggested to constitute monoclonal lesions that can arise in a polyclonal background such as that of hyperplasias (1). In addition to adenomas, a transformation from polyclonal to monoclonal growth of parathyroid tissue probably occurs in most tissues sampled from uraemic patients (12). Changes in cell cycle control result in genetic instability which may lead to clonal proliferation and, in this way, precede tumorigenesis. In the present study, using Ki-67 immunostaining which reliably reflects the proliferating status of cells, we noticed that the proliferative configuration of hyperplastic and adenomatous glands was very similar. Thus the pathological distinction between parathyroid neoplasia and either primary or secondary hyperplasia does not appear to be aided by proliferation rate.

It is a fact that in secondary HPT, nodular formations are frequent; they are thought to be formed by cells of increased proliferative activity (3). In parallel with Ohta et al. (13), the nodular growth pattern in some of the secondarily hyperplastic cases examined appeared to exhibit increased proliferative activity compared with the diffuse pattern but not at a statistically

Figure 3 Cyclin D1 nuclear overexpression in a parathyroid adenoma (immunoperoxidase stain, ×200).

Figure 4 Loss of Fhit immunopositivity (on the right) in a parathyroid carcinoma (immunoperoxidase stain, ×100).
significant level: thus, clonal neoplastic outgrowth may occur on the background of diffuse proliferation apart from that of nodular proliferation. In parallel with Wang et al. (4), in the samples examined, no obvious differences were noticed in the proliferative (or apoptotic) index among various cell types of the diseased or normal parathyroid glands were composed; therefore, proliferative rate does not seem to correlate with parathyroid hormone-synthesizing abilities, which vary considerably among chief, transitional oxyphilic and oxyphilic cells. With regard to Ki-67 immunoreactivity, the only positive finding of the present study was the detection of consistently higher proliferative rates in neoplasms and hyperplasias compared with normal parathyroid glands and the residual rims of normal tissue outside the adenomas; the latter showed very low proliferation rates and this finding makes the enhanced proliferation rate a distinguishing feature of both adenomatous and hyperplastic lesions of the parathyroid gland.

Overexpression of the cyclin D1 oncogene (PRAD1) seems to contribute to parathyroid sporadic tumorigenesis (6), since cyclin D1 immunopositivity was observed in a large proportion of adenomas as well as in both carcinomas of the present study in a considerable proportion of neoplastic cells (mean 27.35%). Cyclin D1 protein is a critical regulator of G1 phase progression in the cell cycle and its overexpression in parathyroid neoplasms appears to be a unifying consequence of all cyclin D1 gene rearrangements and can, therefore, be examined in order to identify more comprehensively tumours in which cyclin D1 is pathogenetically important (11). It is noteworthy that cyclin D1-positive cells were detectable in a considerable proportion of hyperplastic glands, although at lower percentages of parathyroid cells in comparison with tumours. In contrast, in normal parathyroid tissue, cyclin D1 protein was minimal; its participation in the cell cycle control of normal parathyroid growth (14) thus remains questionable.

To the best of our knowledge, this is the first study in which detection of apoptotic cells in parathyroid tissues was performed with an antibody specifically reactive with ssDNA; in detail, this antibody reacts specifically with deoxycytidine and requires stretches of ssDNA of at least 25–30 bases in length for the binding (15). Importantly, in contrast to the TUNEL method (1, 4, 12), monoclonal antibodies to ssDNA are considered specific for apoptotic cell death and do not detect necrotic cells. It is of interest that in neoplastic and secondarily hyperplastic glands apoptotic indexes were found to be generally higher than proliferative ones, as shown in Table 2, whereas in normal and primarily hyperplastic glands the values of the two indexes were closer. Actually, in normal parathyroid glands, the prevalence of apoptosis has been reported to be compatible with that of Ki-67-positive cells engaged in the cell cycle (3); frequency of apoptosis is very low, as expected for low-turnover endocrine tissues in general (5). Primarily hyperplastic glands were found to demonstrate significantly more apoptotic cells than normal controls but at the same time significantly fewer apoptotic cells than tumorous and secondarily hyperplastic glands; of course, the latter groups demonstrated much higher apoptotic indexes than normal controls. Relatively reduced apoptosis (as detected in our primarily hyperplastic cases) could be pathogenetically involved in the promotion of primary hyperplasia by allowing the accumulation of dividing cells. In conjunction with appropriate clinical information, this difference in apoptotic rate between primary hyperplasias and tumours might serve as a useful adjunct to conventional histological techniques in the pathological evaluation of these parathyroid lesions.

Besides primary hyperplasia, other causes of HPT (i.e. tumours and secondary hyperplasia) were associated with a much higher increase in apoptosis. This compensatory increase has been reported previously (1, 4, 12). Interestingly, as far as secondary hyperplasia is concerned, the uraemic state appears to stimulate apoptosis in other cell types apart from parathyroid cells (3). In contrast to primary hyperplasia, in secondary parathyroid hyperplasia, both systemic and local factors have been reported to contribute to an acceleration of programmed cell death in parathyroid tissue, such as disturbed vitamin D metabolism and increased cytoplasmic calcium concentration (12). Whether the increased apoptosis in hyperplastic parathyroid tissue of uraemic patients is linked to the pathological process per se or represents a more general feature of the state of chronic renal failure remains to be seen. From the tumorous and secondarily hyperplastic cases examined, it looks as though the enhancement of parathyroid tissue apoptosis more than compensates 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. Definitive conclusions on the significance of the apoptotic index can be made only after comparative examination of diseased glands of similar size (in other words, of glands that are at similar stages in the natural history of each type of parathyroid disease).

As far as the malignant phenotype is concerned, the only difference in the expression of the examined immunohistochemical markers was noticed in Fhit protein immunostaining. In the two carcinomas in our study, the numbers of Ki-67-positive and apoptotic cells were similar to those of adenomas. In the two carcinomas, a reduction of Fhit protein expression was selectively noticeable; in all other samples, Fhit protein was constantly expressed in parathyroid cells, either normal, hyperplastic or neoplastic. Reduction or loss of Fhit protein expression in human cancer indicates altered FHIT transcripts and genomic FHIT alterations.
and may provide important information in lung carcinogenesis (7); in this cancer, the reduction of Fhit protein expression may be related to overproliferation and suppression of apoptosis (16). It is noteworthy that both carcinomas examined were found to overexpress cyclin D1. This overexpression has been reported to be strikingly frequent in parathyroid carcinomas (17); nevertheless, it remains to be determined whether this feature is causative or represents an epiphenomenon.

In conclusion, cell proliferation and apoptosis are upregulated in all parathyroid lesions and cyclin D1 is overexpressed in a considerable proportion of parathyroid tumours. Tumorous glands exhibit significantly higher apoptotic rates than primarily hyperplastic glands. The role of the FHIT gene in parathyroid carcinogenesis is worth investigating in a large series of parathyroid carcinomas, since its protein expression was found to be decreased specifically in the two carcinomas examined.

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References


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