Differentiation of human parathyroid cells in culture

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Abstract

Continuous culture of parathyroid cells has proven difficult, regardless from which species the cells are derived. In the present study, we have used a defined serum-free low calcium containing medium to culture human parathyroid cells obtained from patients with parathyroid adenomas due to primary hyperparathyroidism. No fibroblast overgrowth occurred, and the human parathyroid chief cells proliferated until confluent. After the first passage the cells ceased to proliferate, but still retained their functional capacity up to 60 days, demonstrated by Ca^{2+} -sensitive changes in the release of parathyroid hormone (PTH) and as adequate cytoplasmic calcium ($[\text{Ca}^{2+}]_i$) responses to changes in ambient calcium as measured by microfluorimetry. Low calcium concentrations enhanced, and vitamin D₃ and retinoic acids (RA) dose-dependently inhibited cell proliferation during the first passage, as determined by [³H]thymidine incorporation, immuno-

histochemistry for proliferating cell nuclear antigen and cell counting. Signs of differentiation were present as the set-points, defined as the external calcium concentration at which half-maximal stimulation of $[\text{Ca}^{2+}]_i$ (set-point_c), or half-maximal inhibition of PTH release (set-point_p) occur, were higher in not proliferating compared with proliferating cells in P0. Inhibition of cell proliferation was accompanied by signs of left-shifted set-points, indicating a link between proliferation and differentiation.

The results demonstrate that human parathyroid chief cells cultured in a defined serum-free medium can be kept viable for a considerable time, and that signs of differentiation occur after proliferation has ceased. The low calcium stimulated cell proliferation may also be inhibited by vitamin D and RA.

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Introduction

The parathyroid glands are the primary regulatory organs for maintenance of serum calcium concentration within a narrow physiological range (Brown 1991). The parathyroid chief cells are equipped with a cell surface-bound calcium-sensing mechanism, which regulates parathyroid hormone (PTH) release (Brown 1991). Abnormal parathyroid cells in parathyroid adenoma, hyperplasia or cancer, are characterized by decreased sensitivity to extracellular calcium concentrations, yielding inappropriate inhibition of PTH release, increased serum PTH levels and hypercalcemia (Hellman *et al.* 1994). This circumstance has been attributed to reduced cell surface expression of calcium sensing receptors (Juhlin *et al.* 1988, Kifor *et al.* 1996). Most documentation of calcium sensing in parathyroid cells relates to the 120 kDa 'calcium sensing receptor', CaR (Brown *et al.* 1993). However, even though both CaR protein and mRNA levels are reduced in parathyroid adenomas compared with normal glands of the same patient, no correlation between the reduction in CaR expression and serum Ca^{2+} or PTH levels has been

found (Farnebo *et al.* 1997). There may also be other calcium receptors, e.g. the 550 kDa glycoprotein LRP2/megalin which has been proposed as expressing a calcium sensing function (Juhlin *et al.* 1987, Christensen *et al.* 1992, Hjälm *et al.* 1996).

The reduced calcium sensing function as well as calcium receptor expression seen in adenomatous and hyperplastic cells have been attributed to various degrees of functional dedifferentiation (Juhlin *et al.* 1988, Kifor *et al.* 1996). Bovine parathyroid cells seem to present a model for this phenomenon, since they dedifferentiate during culture up to 5–7 days, whereby expression of CaR is reduced, and the calcium sensing function gradually impairs (Kifor *et al.* 1996). However, success with continuous culture of bovine cells has in our (and many other) hands been limited by the overgrowth of fibroblasts after 5–10 days, although there are reports in the literature describing successful culture involving an impressive number of doublings (Brandi *et al.* 1986). Also, there seems to be a difference between bovine and human parathyroid cells, since CaR expression in the latter is retained in culture (Roussanne *et al.* 1998).

Vitamin D₃ has an important regulatory role in the parathyroid. Mediated by binding to its receptor (VDR), inhibition of PTH gene transcription and proliferation occurs (Demay *et al.* 1992, Naveh-Manly *et al.* 1995). The effect of vitamin D₃ is the rationale for treatment of hyperparathyroidism secondary to uremia with vitamin D₃ *in vivo*, resulting in lowering of serum PTH levels as well as signs of reduced tumor progression (Slatopolsky *et al.* 1984). In addition, it is evident that parathyroid cells are targets for retinols and retinoic acids, since metabolic conversion of retinol to retinoic acids take place in parathyroid cells, and that these compounds can reduce mRNA levels as well as the release of PTH (MacDonald *et al.* 1994, Liu *et al.* 1996).

To further study the effects of external calcium, vitamin D and retinoids, and their possible relation to the differentiation level in human pathological parathyroid cells, human parathyroid cell cultures are needed. There is only one report documenting successful long-term human parathyroid cultures, and the only cell line reported to be derived from parathyroid cells is of rat origin and does not produce PTH (Sakaguchi *et al.* 1987, Roussanne *et al.* 1998). In this report we describe one model that enables studies of cell proliferation and calcium sensing in human parathyroid cells derived from adenomas cultured in a serum-free medium. For the first time we can demonstrate signs of induced differentiation in parathyroid cells, by coupling increased sensitivity to external Ca²⁺ to reduced proliferation. We also demonstrate effects of vitamin D₃ and retinoic acid on sensitivity to external Ca²⁺ (differentiation) and proliferation of the cultured pathological parathyroid cells.

Materials and Methods

Cell culture

Biopsies of 10 human parathyroid adenomas were obtained during surgery of patients with primary hyperparathyroidism (HPT). This procedure was approved by the local Ethics committee. The patients (7 females, 3 males, mean age 69 ± 12 years) had sporadic non-familial primary HPT, total albumin-corrected serum Ca²⁺ ranging from 2.69 to 3.12 mmol/l (mean 2.82 ± 0.07 mmol/l; reference range 2.20–2.60 mmol/l; orthocresolphthalein method) and intact serum PTH of between 58 and 110 ng/l (mean 72 ± 5 ng/l; reference range 12–55 ng/l; Nichol's Institute, San Capistrano, CA, USA). The glands were minced with scissors after removal of visible fat and connective tissue, and cell suspensions were prepared by digestion in 10 ml (per ≈ 100 mg minced tissue) 1 mg/ml collagenase (Sigma Chemical Co., St Louis, MO, USA), 0.05 mg/ml DNase (Sigma), 1.5% bovine serum albumin (BSA, Sigma) and 1.25 mM Ca²⁺ at 37 °C (Rudberg *et al.* 1986). After digestion in a shaking incubator at 300 r.p.m. for 20–30 min, the suspensions were exposed to 1 mM

EGTA in 25 mM Hepes buffer (pH 7.4) containing 142 mM NaCl and 6.7 mM KCl during 10 s. Dead cells and debris were removed by centrifugation (5 min; 300 × g) through 25% standard isotonic Percoll (Pharmacia-Upjohn, Uppsala, Sweden) diluted in phosphate-buffered saline. Cell viability as determined by Trypan blue exclusion routinely exceeded 95%. This procedure generally yielded small clusters of 2–20 cells, that were cultured in serum-free keratinocyte medium containing 0.09 mM calcium, in which 5 ng/ml epidermal growth factor and 50 µg/ml bovine pituitary extract supplied from the manufacturer (Gibco, Uxbridge, UK) were added. For comparison, parallel batches of dispersed cells were cultured in Dulbecco's modified essential medium (DMEM) (Gibco; ionized calcium concentration 1.8 mM) containing 10% fetal calf serum (Gibco).

Cells were cultured in regular 25 cm² cell culture flasks, in plastic 6-well plates (0.5 × 10⁶ cells/well; Nunclon, Lincoln Park, NJ, USA) for [³H]thymidine incorporation experiments or in plastic 4 cm dishes (1 × 10⁶ cells/dish) for immunostaining. HeLa cells (American Type Culture Collection, Rockville, MD, USA) were used as positive controls in immunohistochemical studies (see below).

[³H]Thymidine incorporation test

After overnight recovery, cells were fed and exposed to different concentrations of calcium (0.09, 1.25 or 3.0 mM), all-*trans*-retinoic acid (atRA; Sigma), 9-*cis* retinoic acid (9cRA; Sigma) or 1,25(OH)₂D₃ (Leo-Pharma, Copenhagen, Denmark) (all compounds added to final concentrations of 10–1000 nM). Vehicle (ethanol) was added in control wells, and all cells were incubated for 48 h, before adding fresh medium containing 1 µCi [³H]thymidine/well. After another 12-h incubation, medium was aspirated and unspecifically incorporated [³H]thymidine precipitated by adding 500 µl 10% trichloroacetic acid for 20 min with the plates on ice. The precipitated material was discarded and the procedure repeated twice, before lysing the cells with 500 µl 0.5 M NaOH and 0.5% Triton X-100/well. Radioactivity was monitored in a β-counter.

Immunohistochemistry

Immunohistochemistry was utilized for identification of cultured cells and characterization of proliferation rate. Immediately after plating and after a defined number of days (up to 60), cells were fixed in 50% acetone for 2 min followed by 100% acetone for another 2 min. For identification of cultured cells, the mouse monoclonal antibody E11 (10 µg/ml) directed against LRP2/megalin, and the rabbit antiserum IHC 610 (1:700; Peninsula Laboratories, Belmont, CA, USA) directed against PTH(1–34), were utilized. Both LRP2/megalin and PTH are highly

expressed in parathyroid chief cells and are absent in endothelial cells and fibroblasts. In addition, based on earlier assumptions that expression of LRP2/megalin is reduced in proliferating and lower differentiated cells, the percentage of LRP2/megalin positive cells was monitored at different stages during culture. For characterization of proliferation rate, cells fixed with 4% formalin for 2 min and ethanol for another 10 min were evaluated for expression of proliferating cell nuclear antigen (PCNA) utilizing the PC10 antibody (1:100, DAKO, Glostrup, Denmark) as described earlier (Yamaguchi *et al.* 1997). In these cultures, after recovery overnight, cells were exposed to 10–1000 nM atRA, 9cRA and 1,25(OH)₂D₃ as above for 48 h. All immunostainings involved usage of the peroxidase–antiperoxidase procedure, i.e. exposure of cells to 1% H₂O₂ for 30 min followed by 10% normal swine or rabbit serum for 30 min. After rinsing in PBS, the cells were incubated with the respective antibody/antiserum for 30 min, followed by a secondary anti-mouse antibody (1:40, DAKO) and peroxidase–antiperoxidase complex (1:250, DAKO) for 30 min respectively. Immunostaining was visualized with 3-amino-9-ethyl-carbazole and counterstaining was performed with Mayer's hematoxylin. Parathyroid chief cells were identified by a cell surface staining of LRP2/megalin and/or cytoplasmic PTH reactivity. For PCNA expression intense nuclear staining was considered positive, and HeLa cells were used as a positive control. The percentage of PCNA positive cells was estimated by two independent observers, blind to the incubation protocol, by calculating the average ratio of stained cells in five randomly chosen areas. Each calculation was performed three times, and there was no significant intra- or interobserver variability.

Cell counting

Five hundred thousand cells were plated and cultured in triplicate with identical additions to the medium as for [³H]thymidine incorporation. Cells were cultured for 48 h, trypsinized and, blind to the incubation protocol, counted in a hemocytometer.

Functional analyses

Investigations of PTH release from plated cells incubated as above and after different durations of culture were performed as previously described (Ridefelt *et al.* 1992). In brief, cells from six different patients were incubated at 37 °C in duplicate in a buffer containing 20 mM Hepes (pH 7.4) at different calcium concentrations (0.5, 1.25 and 3.0 mM) for 1 h. Supernatants were recovered, and PTH(1–34) was analyzed by radioimmunoassay (Ridefelt *et al.* 1992), and intact PTH(1–84) by an immunoradiometric method (Nichol's Institute). Analyses of PTH were correlated to the amount of cells by determining the total protein amount in each well (Bio-Rad, Stockholm,

Sweden). PTH release at 0.5 mmol/l was set to 100%, and maximal inhibition of PTH release was seen at an external Ca²⁺ concentration of 3.0 mmol/l, i.e. further increases in external Ca²⁺ did not additionally reduce PTH secretion in these cells. The external Ca²⁺ concentration at which 50% of the reduction of PTH release between 0.5 and 3.0 mmol/l occurred was defined as set-point_p.

Cells were also cultured on glass cover-slips and incubated with fura-2 acetoxymethylester for 30 min at 37 °C, as described (Johansson *et al.* 1987). The concentrations of intracellular calcium ([Ca²⁺]_i) were determined in small clusters of cells in a dual wavelength fluorescence microscope (Nikon, Tokyo, Japan), with emission at 510 nm and excitation at 340 and 380 nm during various additions of calcium to the perfusion medium.

The external calcium concentration at which half-maximal stimulation of [Ca²⁺]_i occurred (set-point_c) was also calculated as a measure of calcium sensing function and was presumed to correlate to the differentiation state of the cells. In these experiments step-wise increases of external Ca²⁺ from 0.5 to 1.0, 1.5, 2.0, 2.5 and 3.0 mM were performed, and [Ca²⁺]_i calculated at each point (Fig. 1). Since no further increase in [Ca²⁺]_i occurred when increasing external Ca²⁺ to more than 3.0 mM, this was set as the maximum stimulation. Cells were also preincubated with 10–1000 nM vitamin D₃, atRA or 9cRA, alone or in combinations for 2 days, during the P0 and P1 passages, for determination of possible effects on set-points. Additionally 100 or 1000 nM vitamin D₃, atRA or 9cRA were added to fura-2-loaded cells placed in the microfluorimeter in order to document possible acute effects on [Ca²⁺]_i. Control cells were incubated with vehicle alone.

Statistical analysis

Paired, two-tailed Student's *t*-test and ANOVA were utilized, and *P* < 0.05 was considered significant. Values are presented as means ± S.E.M. (standard error of the mean).

Results

Cell culture and basal functions

More than 95% of the dispersed cells consisted of typical parathyroid cells, as revealed by immunostaining with the anti-PTH and/or anti-LRP2/megalin antibodies. Only occasional fibroblasts were seen. After culture in DMEM/10% FCS the ratio of fibroblasts increased to an average of 10% (*n* = 5) after 2 days, 17% after 3 days, and 50% after 5 days and demonstrated the typical spindle shaped appearance, not positive for PTH or LRP2/megalin on immunostaining. During this culture, signs of proliferation of the PTH- and LRP2/megalin-expressing parathyroid chief cells were evident only during the first 1–2 days.

Figure 1 Microfluorimetry of a fura-2-loaded cluster of 4 cells. (A) Set-point_c estimated after step-wise increases of external Ca^{2+} from 0.5 to 1.0, 1.5, 2.0, 2.5 and finally to 3.0 mM (external Ca^{2+} concentration increased 0.5 mM at each arrow). $[\text{Ca}^{2+}]_i$ was calculated at each concentration and the external Ca^{2+} concentration at which 50% increase in $[\text{Ca}^{2+}]_i$ occurred was defined as set-point_c. (B–D) $[\text{Ca}^{2+}]_i$ response of (B) fura-2-loaded cell directly after dispersion, (C) in cell after 2 days in P0, and (D) in cell cultured for 10 days in P1. All cells (B, C, D) were initially incubated in buffer containing 0.5 mM external Ca^{2+} , and the concentration was suddenly increased to 3.0 mM as indicated in each figure. Actual concentrations of $[\text{Ca}^{2+}]_i$ were calculated as indicated in Materials and Methods.

From the third day of culture in DMEM/10% FCS no further increase in numbers of parathyroid cells was detected, which correlated with the time when proliferation of fibroblasts increased dramatically. After 10 days of culture, no LRP2/megalin or PTH positive cells were seen, and virtually all cells were fibroblasts, which proliferated until confluent.

On the other hand, during culture in the serum-free, low calcium-containing medium, most cells were expressing LRP2/megalin and PTH, and only occasional fibroblasts could be detected throughout culture up to 60 days. Even though >95% of dispersed and plated cells demonstrated positive immunostaining with LRP2/megalin and PTH, this had reduced to an average of $82 \pm 12\%$ ($n=5$) after 2 days. There were no statistically significant differences in the ratio of cells positive for LRP2/megalin staining throughout the culture, even in further passages (see below). The cells were viable for up to 60 days in culture, as assessed by Trypan blue exclusion, ability to secrete PTH, and to respond with changes in $[\text{Ca}^{2+}]_i$ upon increases in external Ca^{2+} .

The characteristic Ca^{2+} responsiveness of parathyroid cells was maintained during culture. In acutely dispersed cells, and in those incubated for 2 days in serum-free medium, incubation for 60 min in high external calcium (3.0 mM) inhibited PTH(1–34) secretion by $37.1 \pm 3.8\%$ and $34.5 \pm 3.5\%$ respectively compared with cells incubated with 0.5 mM (Table 1). These numbers were achieved after correlating for the amount of parathyroid cells in each well by determining total protein content of the cells after the 60-min incubation. Similar results were seen when measuring PTH(1–84) concentrations in the medium. Moreover, acutely dispersed cells, as well as cells cultured for 2 days, also responded with a typical transient increase followed by a sustained increase in $[\text{Ca}^{2+}]_i$, when external calcium was raised from 0.5 to 3.0 mM (Fig. 1). All examined cell clusters (acutely dispersed, $n=10$; after 2 days in culture, $n=10$) responded with similar patterns, indicating that this method is sensitive for identification of parathyroid cells.

The cultured cells proliferated, but only in the first flask, during the first passage (P0). Doubling time was estimated

Table 1 Release of PTH(1–34) into the medium during culture at different Ca^{2+} concentrations (first 3 columns), the calculated secreted amounts of PTH(1–34) correlated to total protein concentration of the cells (4th column), calculation of set-point_p (5th column), set-point_c (6th column), the peak $[\text{Ca}^{2+}]_i$ level after a sudden increase of external Ca^{2+} from 0.5 to 3.0 mM (7th column) and the $[\text{Ca}^{2+}]_i$ level of the sustained plateau (8th column) are depicted. Values in acutely dispersed cells, in P0 cells after 2 days culture, in cells in P0 after 2 days of culture with additions of 100 nM vitamin D₃, atRA or 9cRA, alone or in combinations, in P1 cells after 2 days of culture, and in P1 cells after 2 days of culture with additions of 100 nM vitamin D₃, atRA, or 9cRA are presented. The treated P0 and P1 cells are presented together, since no differences occurred between the different groups. Results in columns 2 and 3 are presented as the percentage of release at 0.5 mM external Ca^{2+} , set to 100% (column 1). Numbers of experiments being the basis for each result are indicated

	0.5 mM Ca^{2+} (%)	1.25 mM Ca^{2+} (%)	3.0 mM Ca^{2+} (%)	PTH(1–34) at 0.5 mM	Set-point _p (mM)	Set-point _c (mM)	Peak $[\text{Ca}^{2+}]_i$ (nM)	Plateau $[\text{Ca}^{2+}]_i$ (nM)
Dispersed cells	100 <i>n</i> = 10	85.3 ± 3.5	62.9 ± 3.8	77.3 ± 12	1.47 ± 0.16	1.69 ± 0.35 <i>n</i> = 23	247 ± 12 <i>n</i> = 12	199 ± 8
P0	100 <i>n</i> = 5	88.8 ± 3.9	65.5 ± 3.5	71.1 ± 9	1.53 ± 0.31	1.77 ± 0.33 <i>n</i> = 12	221 ± 13 <i>n</i> = 14	165 ± 22*
P0+ treatment	100 <i>n</i> = 4	82.5 ± 5.3	51 ± 4	72.2 ± 7	1.43 ± 0.21†	1.62 ± 0.41† <i>n</i> = 6		
P1	100 <i>n</i> = 4	81.0 ± 3.2*	52.2 ± 3.9*	53.5 ± 7	1.33 ± 0.40*	1.49 ± 0.14* <i>n</i> = 21	284 ± 8* <i>n</i> = 11	225 ± 11*
P1+ treatment	100 <i>n</i> = 4	79.8 ± 4.1*	55.1 ± 4.2*	57.9 ± 5.9	1.37 ± 0.51*	1.45 ± 0.21* <i>n</i> = 15		

*Indicates significant changes compared to both acutely dispersed and P0 cells; †indicates significant difference compared with untreated P0 cells only.

to be approximately 4 days. However, even though the cells ceased to proliferate when transferred to the second flask, they maintained the ability to respond with the typical biphasic rise in $[\text{Ca}^{2+}]_i$ upon increments in external calcium for up to 60 days of continuous culture (Fig. 1). Calcium also inhibited PTH release in cells during P1, and numbers were adjusted to total protein contents of the investigated cell masses (Table 1). Again, no differences were seen between concentrations of PTH(1–34) and PTH(1–84).

Tendencies towards, albeit not significant, higher set-point_p and set-point_c were seen in P0 cells compared with acutely dispersed cells (Table 1). Moreover, the peak $[\text{Ca}^{2+}]_i$ as well as the sustained plateau $[\text{Ca}^{2+}]_i$ levels after increasing external Ca^{2+} from 0.5 to 3.0 mM were lower in P0 cells compared with acutely dispersed cells. However, cells in P1 passage demonstrated a significant left-shift in their set-point_p, 2 days after plating in this flask (Table 1). Also set-point_c was shifted to the left, and peak and sustained $[\text{Ca}^{2+}]_i$ levels increased during P1. These changes occurred within the first 2 days in P1, and were maintained for the 60 days monitored in this study. Treatment with 100 nM vitamin D₃, atRA or 9cRA for 2 days in P0, alone or in combinations, caused set-point left-shifts, but did not alter set-points after treatment in P1 (Table 1). Since there were no differences between the various treatments in the degree of left-shift, they are presented together in Table 1.

$[\text{Ca}^{2+}]_i$ remained unchanged up to 20 min after adding 100 or 1000 μM 1,25-dihydroxyvitamin D₃, atRA or 9cRA to cells examined directly after dispersion, after 2 days in P0, or after 10 days in P1, and at 0.5 mM external Ca^{2+} .

Effects on proliferation

Proliferation studies were performed during the P0 passage. [³H]Thymidine incorporation demonstrated that low ambient calcium (0.09 mM) stimulated human parathyroid cell proliferation (Fig. 2). Additions of 10–1000 nM atRA, 9cRA and vitamin D₃ were therefore performed at this external calcium concentration. All these compounds dose-dependently inhibited low calcium-stimulated parathyroid cell proliferation, and significant inhibition was achieved at 100 and 1000 nM (Fig. 3). Cell counting experiments as well as immunostaining for PCNA gave similar results as the [³H]thymidine incorporation data (Fig. 3). On average 11.2 ± 7% of the acutely dispersed cells demonstrated intense PCNA immunostaining (Fig. 4). This ratio was increased to 29.8 ± 9% in P0, and reduced to 1.3 ± 1% in P1. A tendency to, albeit not significant, additive effects were seen with combinations of vitamin D₃ with atRA or 9cRA in the PCNA experiments during P0, while this could not be noted in [³H]thymidine incorporation or cell counting experiments (Fig. 3). Concomitantly as proliferation was inhibited, left-shifts in set-points occurred (see above; Table 1).

Discussion

Many attempts to establish parathyroid cells in culture and parathyroid cell lines have been undertaken, but only occasional reports have indicated success (Brandi *et al.* 1986, Roussanne *et al.* 1998) and it has been difficult to repeat some of these data. Most attempts were performed utilizing bovine parathyroid cells, whereas our aim was to culture human parathyroid cells obtained from parathyroid

Figure 2 Effect of calcium on parathyroid chief cell proliferation determined by [^3H]thymidine incorporation. Data are presented as a percentage of radioactivity ($\pm$ S.E.M.) monitored by β -scintillation after incubation at 0.5, 1.25 and 3.0 mM external calcium.

* $P < 0.05$ compared with incubation at 0.5 mM.

adenomas. Based on experience with various growth factor combinations (Ridefelt *et al.* 1995), we tried to optimize the medium for culture of human parathyroid cells. Our results show that a defined serum-free medium originally prepared for keratinocyte culture is suitable for human parathyroid cells, which are found to proliferate until confluent in this medium, although proliferation is hampered by further subculture. In this medium no fibroblast overgrowth occurs, possibly related to the low ambient Ca^{2+} concentration and the serum-free conditions. Parathyroid cells cultured in this medium are viable and respond with adequate changes in $[\text{Ca}^{2+}]_i$ upon alteration of external calcium during the proliferative as well as the quiescent phase. The response is characterized by a transient elevation followed by a sustained rise of $[\text{Ca}^{2+}]_i$ after challenging the cells with a sudden increase in external Ca^{2+} . This response is similar to the one previously reported from bovine and human parathyroid cells (Johansson *et al.* 1987, 1989, Ridefelt *et al.* 1996). We have earlier also demonstrated oscillatory responses in $[\text{Ca}^{2+}]_i$ using a growth cocktail supplemented medium (Ridefelt *et al.* 1995), but such responses do not occur using the present protocol. This further emphasizes how the culture conditions influence the function of parathyroid cells, and the difficulties in interpreting data obtained from parathyroid cell cultures when trying to extrapolate them into the *in vivo* situation. Nevertheless, the present protocol offers a possibility to study proliferating human parathyroid cells which during the culture vary their

differentiation level and express different sensitivity to external Ca^{2+} .

The proliferating P0 cells demonstrated similar set-points as the acutely dispersed cells, while the quiescent P1 cells exhibited left shifts in both set-point_p and set-point_c, as well as increased peak and sustained levels of $[\text{Ca}^{2+}]_i$ after a sudden increase from 0.5 to 3.0 mM external Ca^{2+} . These observations correlate in time with the proliferative state of the cells, i.e. the acutely dispersed cells and the proliferating P0 cells demonstrate similarly right-shifted set-points, while the not dividing P1 cells demonstrate left-shifts. This is further emphasized by the left-shifted set-points, albeit not to a similar degree, in P0 cells incubated for 48 h with vitamin D₃, atRA or 9cRA, where proliferation is also inhibited concomitantly. This phenomenon may reflect a differentiating event in these cells, coupled to the reduced proliferative rate. This observation is the first where signs of increased differentiation occur in parathyroid cells. However, whether the added compounds primarily affect proliferation and whether set-point changes are secondary events, is not clear. One possible explanation for a direct effect on set-points would be increased expression of calcium receptors, CaR and/or LRP2/megalin. Even though LRP2/megalin expression was down-regulated, CaR expression was maintained in human parathyroid cells cultured by Roussanne *et al.* (1998). Our own and other authors' previous data from bovine parathyroid cells indicate that vitamin D₃, atRA and 9cRA do not affect CaR or LRP2/megalin mRNA expression (Rogers *et al.* 1995, Brown *et al.* 1996, Hellman *et al.* 1998). Nevertheless, these compounds may still affect CaR as well as LRP2/megalin expression in human pathological cells. In the present study attempts to measure possible variations in LRP2/megalin expression failed during culture, possibly due to the relatively insensitive immunohistochemical method used. Therefore, to find a molecular explanation for the left-shifted set-point and to discover whether it is a primary or secondary event, *in situ* hybridizations, RNase protection assays or quantitative PCR utilizing probes encoding for CaR and LRP2/megalin may be more informative.

We have previously reported that a certain polymorphic variation in the vitamin D receptor gene is associated with a higher incidence of HPT and right-shifted set-points in postmenopausal women (Carling *et al.* 1995, 1997). Therefore the reduced inhibitory effect of vitamin D₃ may be determined not only by low levels of circulating vitamin D₃, but also by the presence of the polymorphic variant *baT* of the VDR gene. A relative lack of vitamin D₃ may thus occur in some individuals although serum levels of vitamin D₃ are normal. In the present series the inhibitory effect of vitamin D₃ (and retinoids) on PTH release is documented at low external Ca^{2+} concentrations. This implies that at high external Ca^{2+} concentrations such as in HPT, this effect would be of minor importance.

Figure 3 (a) Effect of vehicle alone (A) or addition of 100 nM atRA (B), 9cRA (C), vitamin D₃ (D), atRA and vitamin D₃ (E) or 9cRA and vitamin D₃ (F) on parathyroid chief cell proliferation were determined by [³H]thymidine incorporation. Cells were cultured at 0.09 mM calcium. Data are presented as a percentage of radioactivity monitored by β -scintillation after incubation with vehicle. **P*<0.05 compared with (A). (b) Number of cells remaining after 48 h culture with vehicle alone (A) or the addition of 100 nM atRA (B), 9cRA (C), vitamin D₃ (D), atRA and vitamin D₃ (E) or 9cRA and vitamin D₃ (F). Data are presented as a percentage compared with control wells (A). **P*<0.05 compared with (A). (c) Average percentage of parathyroid chief cells positively immunostained by PCNA antibody after treatment with vehicle alone (A) or addition of 100 nM atRA (B), 9cRA (C), vitamin D₃ (D), atRA and vitamin D₃ (E) or 9cRA and vitamin D₃ (F). **P*<0.05 compared with A.

Nevertheless, in patients with HPT secondary to uremia, levels of serum PTH decrease after initiation of vitamin D₃ treatment (Slatopolsky *et al.* 1984). Increased levels of vitamin D₃ may be needed in patients harboring the *baT* polymorphism in the VDR gene, and upregulation of VDR by vitamin D itself may be another explanation for

the positive effect on PTH release seen at high external Ca²⁺ concentrations.

In accordance with previous *in vitro* and *in vivo* studies (Brandi *et al.* 1986, Naveh-Many *et al.* 1995) we found that low external calcium concentrations (0.5 mM) induced parathyroid cell proliferation, whereas culture

Figure 4 Immunohistochemical staining of cultured parathyroid chief cells, fixed with 4% formalin for 2 min and ethanol for 10 min, and further subjected to a peroxidase–antiperoxidase immunostaining procedure using the PC10 antibody (1:100) against PCNA. Nuclear staining is considered positive. Magnification $\times 150$.

at 3.0 mM ambient calcium significantly reduced cell proliferation. Moreover, we found significant inhibition of cell proliferation resulting in lower cell numbers after treatment with vitamin D₃, atRA or 9cRA. Previous studies have mostly indicated an inhibitory effect of vitamin D₃ on parathyroid cell proliferation (Slatopolsky *et al.* 1984, Szabo *et al.* 1989), although a lack of effect has also been reported (Naveh–Many *et al.* 1995), whereas effects of retinoic acids have not been evaluated.

Retinoic acids bind to cytoplasmic transport proteins and subsequently to nuclear receptors present in parathyroid tissue (Liu *et al.* 1996). The nuclear receptors for vitamin D₃ and retinoic acids (RAR, RXR) form heterodimers, and different effects of these complexes have been documented. Thus, 9cRA binding to an RXR/VDR heterodimer may inhibit or promote the vitamin D₃/VDR effect on genomic transcription rate (Carlberg *et al.* 1993, Schrader *et al.* 1993). It has recently been demonstrated that 9cRA and atRA have similar effects on PTH mRNA expression as vitamin D₃ and thereby suppress PTH release (MacDonald *et al.* 1994, Liu *et al.* 1996). However, this effect may be indirect, since no actual RAR or RXR binding sites seem to be present in the PTH gene promoter region (G Westin, personal communication). As demonstrated in the present study, the effects of vitamin D₃ and retinoids seem to include inhibition of parathyroid chief cell proliferation. Molecular mechanisms for the effect of vitamin D₃ on parathyroid cell proliferation may include the c-myc gene (Kremer *et al.* 1989). Retinoic acids have previously been shown to affect proliferation of many tumor cell lines, and the mechanisms for such effects may be several (Nagy *et al.* 1998, Singh & Lippman 1998).

The present study utilizes a successful protocol with serum-free, low-calcium conditions for culture of human parathyroid cells obtained from adenomas. The previous method of Roussanne *et al.* (1998) utilized cells derived from patients with uremic hyperparathyroidism, and high-phosphate serum containing medium, which also resulted in proliferating human parathyroid cells. Thus, it is possible to obtain dividing human parathyroid cell cultures, which may be used for a number of interesting studies. Our protocol offers studies of parathyroid cells during a dedifferentiated proliferative phase as well as a putatively more differentiated non-proliferative phase. For the first time we can demonstrate correlation between set-point/differentiation level and proliferation in parathyroid cells. Although not the scope of this paper, changes in protein expression may be determined to characterize these events. This study concludes that not only vitamin D₃, but also atRA and 9cRA inhibit chief cell proliferation, and that these compounds may increase calcium sensitivity/induce differentiation during a proliferative phase, measured as increments in set-point_p and set-point_c, as well as inhibit PTH release. Therefore, vitamin D₃, which today is routinely used for the treatment of secondary HPT (or perhaps non-calcemic analogs) may also be used in primary HPT, especially in postmenopausal women. Moreover, retinoids or their analogs lacking unwanted side effects might also be considered for medical treatment of primary as well as secondary hyperparathyroidism.

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