ISOLATION OF HUMAN PARATHYROID CELL TYPE AS A TOOL FOR INVESTIGATING THE MECHANISMS OF HUMAN PRIMARY ALDOSTERONISM

**Coordinatore:** Prof. Gian Paolo Rossi, MD, FAHA, FACC

**Supervisore:** Teresa Maria Seccia, MD, PhD

**Dottoranda:** Abril González Campos

**ANO ACCADEMICO:** 2013/2014
Nessuno deve credere di essere solo, perché in ciascuno vive il sangue di coloro che l’hanno generato, ed è una cosa che va indietro fino alla notte dei tempi. Così siamo solo la curva di un fiume, che viene da lontano e non si fermerà dopo di noi.

A. Baricco
CONTENTS

RIASSUNTO .......................................................................................................................... 7

ABSTRACT ............................................................................................................................ 9

INTRODUCTION ...................................................................................................................... 11

1. PARATHYROID GLANDS ................................................................................................. 11
   1.1 Function .......................................................................................................................... 12
   2.1 Parathyroid Hormone (PTH) ....................................................................................... 13
   2.2 Calcium Sensing Receptor (CaSR) ............................................................................. 15
   2.3 Glial Cells Missing 2 (GCM2) ....................................................................................... 16
   2.4 Chromogranin-A ........................................................................................................... 17
   2.5 Localization of the Markers in the Parathyroid Gland ................................................ 18

3. PARATHYROID HORMONE AND ALDOSTERONE: A LINK BETWEEN SYSTEMS. ................................................................................................................................. 19
   3.1 The Role of PTH in Primary Aldosteronism ................................................................. 23
   3.2 The Role of Aldosterone in Primary Hyperparathyroidism ......................................... 26

4. CELLULAR MECHANISMS UNDERLYING THE LINK BETWEEN ALDOSTERONE AND PTH ................................................................................................................. 28

HYPOTHESIS .......................................................................................................................... 31

MATERIAL AND METHODS .................................................................................................. 33

   Tissue Dispersion ............................................................................................................... 33
   Cell Isolation and Culture .................................................................................................. 33
   Precoating of Magnetic Beads .......................................................................................... 33
   Separation of Parathyroid Cell by Size-Selective Isolation ............................................. 34
   Electron Microscope ........................................................................................................ 34
   Phenotypic Characterization of Cells ............................................................................... 36
RESULTS ........................................................................................................... 41

Primary Cell Culture from Parathyroid Adenoma ........................................... 41

Ultrastructure of Parathyroid Adenoma Cells ................................................... 42

Parathyroid Cell Markers: Gene and Protein Expression ................................... 46

PTH Production .................................................................................................. 53

Mitochondrial Membrane Potential .................................................................. 53

DISCUSSION ........................................................................................................ 57

Primary Cell Culture from Parathyroid Adenoma ........................................... 57

Parathyroid Cell Morphology, Markers Expression and Function ..................... 58

Mitochondrial Membrane Potential .................................................................. 59

Limitations of the Study ..................................................................................... 60

Clinical Relevance .............................................................................................. 60

Perspectives ......................................................................................................... 61

ACKNOWLEDGMENTS ....................................................................................... 63

BIBLIOGRAPHY ................................................................................................. 65
RIASSUNTO

Nelle cellule di paratiroide è espresso il recettore mineralcorticoido. Inoltre, nell’iperaldosteronismo primario i livelli sierici di ormone paratiroideo (PTH) sono aumentati. Questi dati unitamente al fatto che il PTH stimola la secrezione di aldosterone suggeriscono una relazione tra paratiroidi e zona glomerulosa del surrene. L’analisi di tale relazione è stata ostacolata finora dalla mancata disponibilità di una linea cellulare di paratiroide idonea per gli studi in vitro.

Obiettivo. Investigare la possibilità di sviluppare una linea cellulare di paratiroide umana che permetta di investigare se, e attraverso quali meccanismi, la secrezione di PTH sia regolata dall’aldosterone.

Risultati. Dopo digestione di tessuto ottenuto da adenomi di paratiroide sono state isolate le cellule utilizzando due metodi: 1) immunoseparazione mediante biglie incubate con CD90, anticorpo di membrana specifico per i fibroblasti, 2) separazione mediante filtrazione. L’espressione di marcatori propri delle cellule di paratiroide è stata confermata tramite real time qRT-PCR, microscopia elettronica e immunocitochimica nelle cellule in coltura fino al decimo giorno.

Su la base del grado di immunostaining per il PTH e all’intensità di segnale dopo caricamento delle cellule con tetramethylrhodamine-metil-estere (TMRM), una sonda specifica per misurare il potenziale di membrana dei mitocondri, due fenotipi cellulari sono stati identificati.

Conclusioni. Con il protocollo messo a punto nel nostro laboratorio per isolare e coltivare cellule di paratiroide umana si è potuto accertare che le cellule della coltura primaria mantengono il loro fenotipo, caratterizzato dall’espressione dei marcatori propri delle cellule di paratiroide, fino al decimo giorno. Un processo di filtrazione, seguito dall’espressione dei marcatori cellulari e dall’analisi della captazione di TMRM mitocondriale consentono la separazione e l’identificazione delle due sottopopolazioni di cellule che formano il tessuto paratiroideo, cellule ossifile e cellule principali.
ABSTRACT

Background. The detection of the mineralocorticoid receptor in parathyroid cells alongside recent evidences implicating the parathyroid hormone (PTH) in overproduction of aldosterone in primary aldosteronism in spite of suppression of the renin-angiotensin system indicate a relationship between parathyroids and the adrenocortical zona glomerulosa. The investigation of this relationship has been hampered due to lack of parathyroid cell line suitable for in vitro studies.

Objective. To develop a technique to obtain a primary culture of human parathyroid cells with high yield and purity and to obtain subpopulations of chief and oxyphil cells

Results. After digestion of tissue from parathyroid adenomas, we isolated cells using an immunomagnetic bead method to remove fibroblasts and filtration devices to isolate cells by size. real-time qPCR evidenced expression of specific markers of parathyroid cells in cultured cells until day 10. At protein level, we found that some cells showed marked PTH immunostaining, whereas the remaining cells showed only a faint signal, suggesting the presence of two cell types. After loading cells with tetramethylrhodamine-methyl-ester (TMRM), we found two cell types, which differed for the fluorescent signal intensity.

Conclusions. We found an optimal protocol to obtain a culture of human parathyroid cells that includes immunobinding-based technique and cellular filtration. Cultured cells retain their characteristic expression profile until day 10. Analysis of the mitochondrial uptake of TMRM and cell markers allows identification of two cell types forming the parathyroid tissue, i.e. oxyphil and chief cells.
INTRODUCTION

1. PARATHYROID GLANDS

The parathyroids are four small glands located in humans in the cervical region, with one gland behind each of the upper and each of the lower poles of the thyroid. Its main function is the secretion of parathyroid hormone (PTH), a hormone that is essential for the regulation of calcium and phosphate metabolism. Each parathyroid gland is about 6 millimeters long, 3 millimeters wide, and 2 millimeters thick, with an appearance of dark brown fat that renders the glands similar to a lobule of the thyroid gland. For this reason, before the importance of these glands was generally recognized, total or subtotal thyroidectomy frequently resulted in the removal of the parathyroid glands with ensuing hypoparathyroidism. Removal of half parathyroid glands usually causes no major physiologic abnormalities, removal of three glands causes only transient hypoparathyroidism and even a small amount of parathyroid tissue is sufficient to provide enough function to avoid hypoparathyroidism showing the ability of the gland to increase in dimensions and function (1).

The parathyroid gland of the adult contains mainly chief cells, with a small to moderate number of oxyphil cells, but these are absent in young humans and some animals. The chief cells are believed to secrete most, if not all, PTH. However, the oxyphil cells, which are deemed to be modified or depleted chief cells that no longer secrete hormone, may acquire the ability to produce PTH with aging or upon exposure functional stress (1, 2).

Histologically, oxyphil cells are larger than chief cells (12–20 μm vs.6–8μm), with more eosinophilic cytoplasm due to high mitochondrial content. The detection of transitional oxyphil cells, which are more eosinophilic but similar in size to the chief cells, suggests that oxyphil cells derive from chief cells. Additional evidence for a chief cell-to-oxyphil cell transdifferentiation is that both transitional and oxyphil cells express PTH and Glial Cell Missing 2 (GCM2), a parathyroid-specific transcription factor that is essential for parathyroid gland development (2).
1.1 Function

A remarkable feature of the parathyroid glands is sensitivity to small changes in serum concentration of calcium ion \((\text{Ca}^{2+})\), which leads to large changes in PTH secretion. This feature is unique to the parathyroid glands. All other endocrine glands, in fact, increase hormone secretion after exposure to high extracellular \(\text{Ca}^{2+}\) concentrations.

Although the four parathyroid glands are quite small, they are very vascular. As the blood filters through the parathyroid glands, they detect the amount of calcium present in the blood and react by increasing or decreasing PTH synthesis. When the calcium level in the blood is too low, the cells of the parathyroid glands sense it and produce PTH. Once the PTH is released into the blood, it circulates to act in a number of target tissues to increase the amount of calcium in the blood. When the calcium level in the blood is too high, parathyroid cells produce less PTH, allowing calcium levels to decrease. This feed-back mechanism acts constantly, thereby maintaining \(\text{Ca}^{2+}\) (and PTH) in a very narrow "normal" range (1).
2. Parathyroid Cell Markers

2.1 Parathyroid Hormone (PTH)

PTH is synthesized on the ribosomes in the form of a preprohormone, a polypeptide chain of 110 amino acids that is cleaved first to a pro-hormone with 90 amino acids and then to the mature hormone itself comprising 84 amino acids at the endoplasmic reticulum and Golgi apparatus. The final hormone PTH, which finally is packaged in secretory granules in the cytoplasm of the cells, has a molecular weight of about 9,5 KDa. Smaller compounds with as few as 34 amino acids adjacent to the N terminus of the molecule were also isolated from the parathyroid glands, which are functionally active. Because the kidneys rapidly remove the whole 84-amino acid hormone within minutes, but fail to remove many of the fragments for hours, a large share of the hormonal activity can be caused by the fragments (3).

The overall role of PTH is to increase Ca\(^{2+}\) concentration via three different mechanisms (1):

1. **PTH has two main effects on bone in causing absorption of Ca\(^{2+}\) and phosphate.**
   
   One is the *rapid phase* that begins within minutes and increases progressively for several hours. This phase results from activation of the bone cells (mainly the osteocytes) to promote Ca\(^{2+}\) and phosphate reabsorption. The *second phase is much slower one*, requiring several days or even weeks to become fully developed. This is because it results from proliferation of the osteoclasts, followed by increased osteoclastic reabsorption of the bone itself, not merely absorption of the Ca\(^{2+}\) phosphate salts from the bone.

2. **PTH increases renal tubular reabsorption of Ca\(^{2+}\) while diminishing phosphate (PO4) reabsorption.** Moreover, it increases the rate of reabsorption of Mg\(^+\) and H- ions while it bennts the reabsorption of Na, K+, and amino acid as it does for PO4. The increased Ca\(^{2+}\) absorption occurs mainly in the late distal tubules, the collecting tubules, the early collecting ducts, and to a lesser extent the ascending loop of Henle. Such effect is the most relevant. Actually, were it not for the PTH- withdrawal in the kidneys to increase Ca\(^{2+}\) reabsorption, a
continual loss of Ca\(^{2+}\) into the urine would eventually deplete both the extracellular fluid and the bones of this mineral.

3. PTH greatly enhances both Ca\(^{2+}\) and PO\(_4\) reabsorption from gut by increasing the formation in the kidneys of 1, 25-dihydroxycholecalciferol (1,25(OH)\(_2\)D) from 25-hydroxycholecalciferol (abbreviated 25(OH)D) (Fig.1).

**Figura 1: Overview of the PTH Effects**  Decrement in serum calcium levels stimulate PTH secretion by the parathyroid glands (Initial stimulus), which targets the kidney to reduce urinary Ca\(^{2+}\) excretion and enhance the excretion of phosphate (PO\(_4\)), and targets bone to increase the efflux of Ca\(^{2+}\) and PO\(_4\) (Physiological response). The resulting increase in 1,25(OH)\(_2\)D targets the gastrointestinal tract to increase dietary absorption of calcium. The final result is an increase in the amount of Ca\(^{2+}\) in the blood.

*Adapted from ©2013 Pearson Education, Inc*
2.2 Calcium Sensing Receptor (CaSR)

The ability of chief cells of the parathyroid glands to sense small changes in blood ionized Ca\(^{2+}\) levels and trigger the adaptive responses is mediated by the calcium sensing receptor (CaSR), which is located on the cell surface.

The CaSR was the first receptor to be described for which the natural ligand is an ion. It is a member of subfamily C of G protein-coupled receptors (GPCRs), with a large extracellular domain, seven membrane-spanning regions and an intracellular portion that couples to G proteins and other signal transduction pathways. The gene encoding the protein in humans is located on chromosome 3q13.3-21 (4).

The protein CaSR is a member of the C family of G-protein–coupled receptors and, in humans, is 1,078 amino acids in length. The amino-terminal portion of the molecule contains approximately 600 aminoacid residues and forms a very large extracellular domain (5, 6). Clusters of acidic amino acids in this portion of the CaSR are thought to interact with extracellular Ca\(^{2+}\) ions, thereby modulating the levels of receptor activation and signal transduction (7-9). Although calcium ions represent the physiologically relevant ligand for the CaSR in vivo, a number of divalent, trivalent, and polyvalent cations, and various amino acids, also can activate the CaSR in vitro (5).

Decreased expression of both CaSR and vitamin D receptor (VDR) expression has been described in parathyroid adenomas and hyperplastic parathyroid tissues (10-13). It is unknown whether such changes are correlated, but alterations in CaSR expression may contribute to abnormalities in cell cycle and cell growth in the parathyroid tissue. In vivo findings suggest that signaling through the CaSR is largely preserved in primary hyperparathyroidism even modest reductions in CaSR expression (4). Despite to these findings, it is not entirely clear if the reductions in protein expression alone are sufficient to attenuate signal transduction by CaSR in hyperplastic or adenoma parathyroid tissue or whether such changes explain defects in calcium sensing and their impact in clinical conditions.
2.3 Glial Cells Missing 2 (GCM2)

The thymus, thyroid, and parathyroid glands develop from the pharyngeal region in vertebrates, with contributions both from pharyngeal endoderm and from neural crest cells in the pharyngeal arches. Studies of gene knockout mice showed that the hoxa3, pax 1, pax 9, and Eya1 transcription factors are needed to form parathyroid glands as well as many other pharyngeal pouch derivatives, such as the thymus (14). Gunther et al. studied GCM2, a mouse homologue of Drosophila GCM, a transcription factor whose expression is restricted to the parathyroid glands, and showed that GCM2-deficient mice lacked parathyroid glands and exhibited hyperparathyroidism, thereby identifying GCM2 as a master regulatory gene of parathyroid gland development. In contrast, thymus development was not affected by GCM2 deletion. Despite the lack of parathyroid glands, GCM2-deficient mice had PTH serum levels similar to those of wild-type mice (in the presence of hypocalcemia), as did parathyroidectomized wild-type mice. Expression and ablation studies identified the thymus, where GCM1, another GCM homologue, is expressed as the additional, downregulatable source of PTH. Thus, GCM2 deletion uncovered an auxiliary mechanism for the regulation of calcium homeostasis in the absence of parathyroid glands. A patient with a defective GCM B gene, the human equivalent of GCM-2, exhibited hypoparathyroidism and complete absence of PTH from the bloodstream (15, 16).
2.4 Chromogranin-A

The chromogranins (Chromogranin-A and Chromogranin-B), secretogranins (type I and II) and additional related proteins (7B2, NESP55, proSAAs, and VGF) that together comprise the Granin Family, play essential roles in regulating the secretory pathway that are responsible for controlled delivery of peptides, hormones neurotransmitters and growth factors (17).

Granins are relatively abundant acidic proteins that localize in secretory vesicles, where they bind \( \text{Ca}^{2+} \) and form aggregates. Endocrine, neuroendocrine, and neuronal cells secrete a variety of peptides and hormones via \( \text{Ca}^{2+} \)-dependent release. The number of dense core secretory granules in an endocrine cell (~10,000) together with the high \( \text{Ca}^{2+} \) binding capacity of resident granin proteins and their abundance (~2-4mM), constitute a major intracellular calcium reservoir (18,19).

Chromogranin-A (CGA), also referred to as secretory protein-I, is a 50-kDa protein synthesized and secreted by most endocrine cells together with the native hormone (20). It appears to be the major secretory protein in the parathyroid secretory granules (21) and, therefore, can be considered a marker of parathyroid adenoma (22).
2.5 Localization of the Markers in the Parathyroid Gland

Immunohistologic studies performed in the parathyroid glands of chronic kidney disease (CKD) patients showed higher expression of CaSR and PTH in oxyphil cells, whereas both oxyphil and chief cells express the transcription factor, GCM2 (1) which is critical for the parathyroid development and expression of PTH and CaSR (23). GCM2 was heterogeneously expressed, with the highest expression in the oxyphil clusters and the lowest in the chief cell nodules, suggesting it may be responsible, in part, for the higher expression of PTH. PTH was modestly, although significantly, higher in oxyphil clusters than the diffuse chief cells while CaSR expression was slightly, but significantly, higher in oxyphil clusters and nodules than in chief cells, whereas the VDR was not differentially expressed (Table 1). The authors concluded that oxyphil cells from CKD patients express parathyroid markers found in chief cells, strongly suggesting that oxyphil cells are not simply deactivated chief cells. The presence of VDR and CaSR suggest that oxyphil cells release PTH in a regulated fashion and this fact could be relevant in the interconnection of parathyroid glands with other tissues (1).

**Table 1: Cellular Characteristic of Parathyroid Tissue**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Chief cells</th>
<th>Oxyphil cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size</td>
<td>6 - 8 μm</td>
<td>12 - 20 μm</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>Eosinophilic cytoplasm</td>
<td>Cytoplasm more eosinophilic (mitochondrial content)</td>
</tr>
<tr>
<td>Parathyroid markers</td>
<td>PTH +</td>
<td>PTH +</td>
</tr>
<tr>
<td></td>
<td>GCM2 +</td>
<td>GCM2 +</td>
</tr>
<tr>
<td></td>
<td>CaSR +</td>
<td>CaSR ?</td>
</tr>
<tr>
<td></td>
<td>VDR +</td>
<td>VDR ?</td>
</tr>
</tbody>
</table>

3. PARATHYROID HORMONE AND ALDOSTERONE: A LINK BETWEEN SYSTEMS.

Experimental data published by Martin, et al. in early 70s provided the evidence of an interaction between the adrenal and parathyroid glands. The Authors investigated electrolyte excretion in rats adrenalectomy (ADX), parathyroidectomy (PTX) or both (ADX-PTX) before and during administration of aldosterone, parathyroid extracts, or both hormones. Increased urine Na\(^+\) and Cl\(^-\) and decreased K\(^+\) were found in the ADX-PTX rats compared to the other groups. Aldosterone caused a decrease in all groups in urine Na\(^+\), Cl\(^-\) and PO\(_4\)\(^-\), except, and in stool Ca\(^{2+}\) in ADX-PTX rats, along a rise in urine K\(^+\). Parathyroid extracts, in contrast, caused an increase in urine Na\(^+\) and Cl\(^-\) in all groups except the ADX-PTX rats, and in urine and stool PO\(_4\) and Ca\(^{2+}\) in all groups. The administration of the both hormones caused a similar decrease in urine Na\(^+\) but much higher urine K\(^+\) and Cl\(^-\) than with aldosterone alone. Phosphaturic effect parathyroid extracts was blocked by addition of aldosterone, but little effect was found on stool PO\(_4\) and urine and stool Ca\(^{2+}\). Addition of aldosterone reversed these effects, which appeared related to presence or absence of parathyroid glands. This study suggested a complex interrelation between adrenal and parathyroid glands that involve electrolyte excretion (24), can be considered the seminal study that 'inspired' all subsequent and more indeeping studies.

Later, many data suggested that deregulation of aldosterone or PTH can play an important role in the development and progression of cardiovascular disease. However, the mechanisms underlying the interactions between aldosterone and PTH remained unknown (25).

Our group recently demonstrated the presence of Mineralocorticoid Receptor (MR) in parathyroid-secreting cells in the PTH-secreting adenoma and in the normal parathyroid gland (Fig. 2); another hallmark result was the expression of PTH receptors in the aldosterone-producing adenoma (APA) tissue and in surrounding satellite nodules of the tumor (26). The activated MR–aldosterone complex regulates transcription of numerous genes in a tissue-specific pattern. Experimental and clinical studies documented that aldosterone mediated inflammatory and fibrotic effects were associated with left
ventricular hypertrophy and reduced kidney function (27). Tomaschitz and others recently reported an independent association between plasma aldosterone levels within the ‘normal range’ and cardiovascular mortality, in particular fatal stroke and sudden cardiac death (28-30).

**Figure 2:** A-B. Representative sections of human parathyroid gland incubated with a specific antibody against the human mineralocorticoid receptor (MR) showing intense nuclear staining and a weaker cytoplasm staining. C. A section of normal human kidney was similarly exposed to the same antibody as a positive control shows a strong cytoplasmic expression in the tubules and the lack of staining in glomeruli. D. Agarose gel electrophoresis of MR gene amplicon from the parathyroid adenoma of index case (lane 2) and a normal human parathyroid (lane 3) shows the transcript of this gene in these tissues. Untranscribed RNA from normal human parathyroid (lane 4) and water served as negative controls (lane 5).

*Adapted from Maniero C et al Hypertension 2011;58:341-346.*
The identification of PTH receptors within the cardiovascular system (cardiomyocytes, vascular smooth muscle, and endothelial cells) indicates that inappropriate PTH secretion may impact on the cardiovascular (CV) health. Administration of PTH after myocardial infarction attenuates ischemic cardiomyopathy by increasing migration of bone marrow-derived stem cells to the ischemic myocardium (31). On the other hand the PTH excess in primary hyperparathyroidism (PHPT) is linked in the long-term to adverse effects as bone loss and increased fracture risk, coronary microvascular dysfunction, derangement of lipid and glucose metabolism, subclinical aortic valve calcification, increased aortic stiffness, endothelial dysfunction and arterial hypertension (32–38). Accordingly, patients with PHPT have a remarkably higher risk to die from CV causes compared with the general population (39, 40). Various observational linked higher levels of PTH even in the absence of PHPT to an increased risk of CV morbidity and mortality (41–47). Elevated PTH levels in patients with a declining kidney function have been related to soft tissue calcifications and subsequent adverse cardiovascular diseases (CVD) outcome (48, 49). Figure 3 shows a schematic representation of the interplay between PTH and aldosterone hormone.
Introduction

Figura 3: Overview of the mechanisms and cardiovascular impact resulting from the interaction of aldosterone and parathyroid hormone. Inappropriately elevated aldosterone (ALD) secretion results in accelerated renal (and fecal) calcium and magnesium loss and salt/water retention and increased parathyroid hormone (PTH) secretion via activating PTHR and MR. Dietary salt loading pronounces aldosterone related renal cation loss. MR-mediated hypocalcemia (hypomagnesemia) further stimulates PTH secretion (via activating the calcium sensing receptor (CaSR). PTH induces myocardial damage via stimulating calcium influx in cardiomyocytes resulting in an intracellular and mitochondrial calcium overload and reduction in intra-mitochondrial ATP levels contributing to a disturbed redox status and increased oxidative stress. Aldosterone excess exerts genomic and non-genomic profibrotic and proinflammatory effects on blood vessels and the myocardium. Adapted from Tomaschitz A, et al. Metabolism 2014;63(1):20-31.
3.1 The Role of PTH in Primary Aldosteronism

Primary aldosteronism (PA), is characterized by chronic elevation in plasma aldosterone, is the most common endocrine cause of secondary arterial hypertension (50). The term ‘primary’ implies that the underlying mechanisms are currently unknown. The prevalence of PA was found to be 11.2% in the hypertensive patients referred to a specialized center for high blood pressure and can be estimated to be 17–23% in drug-resistant hypertension. Several large cross-sectional and prospective studies showed a relationship between circulating aldosterone levels and mortality risk (26, 50–57), thereby providing evidence that aldosterone causes organ damage and enhances the cardiovascular risk. Absolute aldosterone excess is in fact strongly associated with a higher risk of development and progression of left ventricular hypertrophy, coronary artery disease, sudden cardiac death, chronic kidney disease and stroke (57-59).

The classical view that aldosterone acts exclusively on the electrolyte transport in epithelial cells has been broadened after the MR has been identified in non-epithelial cells, thereby providing experimental support to the aforementioned studies (26, 50–57). Moreover, in addition to the classical genomic effects, non-genomic aldosterone-mediated effects have been identified in various tissues outside of the kidneys and colon, e.g. endothelial cells and cardiomyocytes (60,61), further supporting a role of aldosterone in mediating organ damage. However, novel mechanisms are emerging.

Recent evidences, actually, strongly suggest that PTH may sustain or trigger the excess production of aldosterone in PA despite the suppression of the renin-angiotensin system, the high blood pressure and hypokalaemia, all factors that would be expected to shut off aldosterone secretion (61, 62). One study performed by our group showed that patients with PA had significantly higher plasma PTH than patients with primary (essential) hypertension, even being characterized by comparable urinary Ca²⁺ excretion and similar deficient 25(OH) vitamin D. Of great interest, adrenalectomy normalized PTH levels and increased ionized Ca²⁺ in APA patients (63) (Fig.4).
In another study from Italy it was observed that the significantly high serum concentrations of intact PTH found in 120 PA patients decreased after 1 month of MR blockade with 100 mg spironolactone daily, along an increase in serum-ionized calcium (64), thus further supporting the contention that PTH plays a role in PA.

The higher plasma PTH levels found in PA patients than in essential hypertension could also have a clinical impact: A study from our laboratory showed that PTH levels are elevated in PA patients with an APA and much less as in those with bilateral adrenal hyperplasia (BAH). Hence, it was proposed that raised serum PTH levels can be useful for selecting the PA patients to be submitted to adrenal vein sampling (AVS) and for discriminating between subtypes of PA (65).

Finally, given the emerging evidence implicating hyperparathyroidism as a cardiovascular risk factor, it could be arguable that the high serum PTH found in PA patients contributes to the excess cardiovascular damage (Fig. 5) (26).
Introduction

Figure 5. Schematic representation of the possible bifunctional link between the adrenocortical zona glomerulosa and the parathyroid gland. Given the secretagogue effect of PTH on aldosterone, excess secretion of PTH can cause hyperaldosteronism, which in turn can modulate the secretion of PTH. Excess levels of PTH and aldosterone can have a detrimental effect on the vascular wall, resulting in vascular damage.

3.2 The Role of Aldosterone in Primary Hyperparathyroidism

Primary hyperparathyroidism (PHPT), the third most common endocrine disorder, is characterized by excess PTH secretion, which is inappropriate with respect to the prevailing concentration of ionized calcium or to increased secretory cell number (66). In the long term, PHPT is associated with the development of osteoporosis and fracture risk, and higher risk of hypertension, left ventricular hypertrophy, arrhythmia, diabetes, hyperlipidemia, and, most importantly, cardiovascular morbidity and mortality (67-70).

Evidence for a bidirectional link between aldosterone and PTH in humans had initially been derived mainly from case reports. The first report was a patient with primary hyperparathyroidism and findings consistent with primary hyperaldosteronism: Elevated levels of plasma aldosterone, which could not be suppressed by a high sodium diet alone or in combination with fludrocortisone, a decline of the elevated plasma aldosterone levels after 4 h of deambulation and low PRA, which was unresponsive to stimulation by a low sodium diet coupled with diuretic-induced volume depletion and 4 h of deambulation. Removal of a parathyroid adenoma not only normalized Ca\(^2+\) and PTH, but also improved blood pressure, hypokaliemia, and aldosterone levels. Since then, the role of PTH on aldosterone metabolism has remained controversial (71).

In this context, our group also described a case report with a combination of unrecognized PA and resistant hypertension in an adult patient with evidence of hypertension-related target organ damage and a history of cardiovascular events. The correction of PA with adrenalectomy and the development of hyperparathyroidism suggested the occurrence of a functional link between the adrenocortical zona glomerulosa and the parathyroid gland, which was totally unsuspected. The patient’s blood pressure first responded dramatically to adrenalectomy and then responded further to parathyroidectomy (26).

Several cross-sectional and prospective studies also documented a strong relationship between aldosterone levels and arterial hypertension as well as increased arterial
stiffness. In view of the interaction between aldosterone and PTH, one might speculate that the interplay between both hormones aggravates blood pressure elevation, blood vessels remodeling and cardiovascular disease in patients with elevated PTH (72,73). The interplay between PTH and aldosterone is increasingly suggested as an important mechanism underlying the increased risk of cardiovascular damage observed in PHPT. To explain the biochemical changes following parathyroid surgery, it has been suggested that hyperaldosteronism might be caused (directly or indirectly) by PHPT and vice versa (74-76).

In summary, the observations that in patients with PHPT: (1) plasma aldosterone and PTH concentrations were positively correlated; (2) at multivariate analysis, preoperative PTH was an independent predictor of plasma aldosterone concentration; (3) PTH levels >100 ng/L were an independent predictor of abnormally elevated plasma aldosterone concentration, are consistent with the concept that PTH plays a role in triggering or maintaining aldosterone secretion in vivo (77,65).
4. CELLULAR MECHANISMS UNDERLYING THE LINK BETWEEN ALDOSTERONE AND PTH

Several experimental studies were performed with the aim of clarifying the mechanisms underlying the effect of PTH on aldosterone secretion from the adrenals. It is well known that PTH stimulates the entry of cytosolic $\text{Ca}^{2+}$ into the mitochondrial matrix and this step is essential for the initiation of steroidogenesis within the mitochondria (78-80). However, it is still under investigation whether PTH stimulates adrenal aldosterone synthesis directly.

Olgaard et al. evaluated the effect of PTH on $\text{Ca}^{2+}$-mediated aldosterone secretion in isolated rat adrenocortical zona glomerulosa (ZG) cells. Aldosterone release increased significantly by up to 200% above baseline values in cells exposed to PTH (1–84) and PTH (1–34), thereby suggesting that PTH exerts $\text{Ca}^{2+}$ ionophore-like effects in the ZG causing increased $\text{Ca}^{2+}$ stimulated aldosterone secretion (81).

Mazzocchi et al. demonstrated that in dispersed adrenocortical cells PTH and PTH-related protein increase aldosterone production by binding to the PTH/PTH-rP receptor, activating cellular adenylate cyclase/cAMP-dependent protein kinase, phospholipase C/protein kinase C- and cAMP-dependent signaling cascades (82). In this context Maniero et al. found that both APA and nodules detected in adrenal hyperplasia expressed the type 1 PTH receptor at both the mRNA and the protein level. A similar PTH receptor expression was found in other cases of PA that did not show hyperparathyroidism but only a subtle elevation of PTH plasma levels. These findings could explain why human dispersed adrenocortical cells responded with aldosterone and cortisol release to either PTH or the PTH-related peptide.

Another important finding contributing to support the mechanistic link between adrenal gland and parathyroid glands is the expression of MR in parathyroid cells (Fig. 1) (26). In epithelial tissues, MR activation by cortisol is mainly prevented by the cortisol-inactivating enzyme 11$\beta$-hydroxysteroid dehydrogenase-2. In the setting of increased generation of reactive oxygen species, e.g. in chronic kidney disease and heart failure, cortisol might also activate the MR—in addition to aldosterone—thus aggravating
Introduction

profibrotic and proinflammatory effects (83, 84). To date it is unclear, however, whether cortisol affects renal handling of Ca\textsuperscript{2+} via binding to the MR. One recent study revealed an upregulated expression of PTH-related peptide in the mice kidney after 4 weeks treatment with cortisol. Moreover, in a small cohort of patients with PHPT, circulating cortisol levels decreased significantly after parathyroidectomy (85, 86). Conversely, intravenous infusion of PTH in healthy adults increased plasma cortisol concentration (87). Since 1) hypercalcaemia, caused by PTH excess, results in a transient rise of adrenocorticotropic hormone secretion; 2) PTH stimulates steroid hormone synthesis in part by binding to the adrenocorticotropic hormone receptor; 3) cortisol upregulates PTH-related peptide, one might speculate that this sequence may impact on cardiovascular health. In this perspective the conceivable relationship between glucocorticoids and the steroid-related marker 11\textbeta-hydroxysteroid dehydrogenase-2 with PTH remains to be addressed (62).
HYPOTHESIS

The recent detection of the MR in parathyroid cells (26) suggeststhat PTH plays a role in maintaining overproduction of aldosterone in PA in spite of suppression of the renin-angiotensin system, thereby suggesting a relationship between parathyroid glands and the adrenocortical zona glomerulosa.

The investigation in this field of this relationship has been hampered thus far by the lack of a parathyroid cell line suitable for in vitro studies. Therefore, we planned to develop a technique to obtain a primary culture of human parathyroid cells with high yield and purity and to obtain subpopulations of chief and oxyphil cells.

Primary Objective

To develop a human parathyroid cell line that can be used for investigating the mechanisms underlying the effects of aldosterone on PTH synthesis and secretion.

Specific Aims

1. To develop a protocol to obtain a pure primary culture of human parathyroid cells.

2. To develop a protocol to separate and collect two cell types, e.g. chief and oxyphil cells, that constitute the parathyroid tissue.

3. To evaluate the gene and protein expression levels of the markers specific for parathyroid cells in the separated oxyphil and chief cells.

4. To evaluate PTH synthesis and production in the primary cultures.
MATERIAL AND METHODS

Tissue Dispersion

Tissue samples were obtained in the operating room from patients undergoing surgery for parathyroid adenoma. After removal of visible fat and capsule the glands were minced and then treated by digestion in 1 mg/ml collagenase type II, 0.05mg/ml DNAse I (Roche; Milan Italy) in RPMI-1640 media (Sigma-Aldrich®, Italy) for 2 cycles of 20 minutes at 37°C, followed by mechanical disaggregation (GentleMACS™ Dissociator by Miltenyi Biotec). Cells were filtered through a 40 mm cell strainer, and then centrifuged for 5 min at 800x g. Their viability was assessed by trypan blue exclusion.

Cell Isolation and Culture

Cell suspension obtained after enzymatic and mechanic digestion was washed with RPMI-1640 medium (Sigma-Aldrich®, Italy) and then incubated with CD90 coated magnetic beads by gentle shaking for 30 min at 4 °C. Beads were used at a ratio of five beads per cell. After separating the bead-bound CD90 positive cells with a magnet, CD90 negative cells were cultured at a density of ~1x10^5 cells into 75 cm^3 tissue culture flask (Nunclon Delta Surface Thermo Scientific) or six well plate (Multiwell™ 6 well Becton Dickinson Labware) and maintained in a humidified 5% carbon dioxide/air atmosphere at 37 C. Fresh medium was added every 48 hours.

Precoating of Magnetic Beads

To eliminate fibroblast contamination, magnetic beads (Dynabeads; Invitrogen, Milan, Italy) (1x10^7/25μl) conjugated with goat anti-mouse IgG were washed three times with PBS/0.1% BSA and then precoated with 1.5 μg mouse monoclonal antibody against human CD90 (Thymus cell antigen 1, an anchored membrane protein express in fibroblast cell membrane). Pre-coating was obtained by incubating the beads with the primary mouse antibody overnight at 4 °C under rotation. Beads were washed three times with PBS/0.1% BSA and then used for cell separation by depletion of fibroblast cells.
Material and Methods

Separation of Parathyroid Cell by Size-Selective Isolation

The isolation of cells was performed with Screencell Cell Culture Kit ® (Screencell Technology USA) with a protocol modified for tissue samples. The ScreenCell® filtration devices were developed in order to isolate circulating tumour cells by size on a microporous membrane filter, where circular pores are calibrated 7.5±0.36 μm or 6.5±0.33 μm and randomly distributed throughout the filter (88).

**a. Filtration.** After tissue digestion, cells were suspended in RPMI media with antibiotic (4-6 ml depending of the sample size), and then added 1 ml of ScreenCell LC dilution buffer for 2 minutes in order to lyse red blood cells. The reaction was stopped by adding 1.6 ml culture medium. The sample filtration was performed according to the producer's instruction.

**b. Cell Culture.** The filter with cells was cultured in a 24-well tissue culture plate (Multiwell™ 24 well Becton Dickinson Labware) with RPMI-1640 medium 5% FBS while the supernatant, or filter cells, was obtained by centrifugation 5 minutes at 4,2 min⁻¹ or by dilution in small volume of culture media. All cell types were used within 10 day of culture for all experiments (Fig.6).

Electron Microscope

For electron microscopy studies, oxyphil and chief parathyroid cells were separated with ScreenCell®Cyto filters and then cultured in 24-multi-well insert systems treated with Matrigel® (BDBiosciences, Australia). Classic primary culture were maintained in standard 24-well tissue culture plate (Multiwell™ 24 well Becton Dickinson Labware) for 5-12 days.

Monolayers of parathyroid cells were fixed in 3% in glutaraldehyde (Serva Electrophoresis- Heidelberg, Germany) in 0.1M phosphate buffer, post-fixed in 1% osmium tetroxide (Agar Scientific Elektron Technology, UK) in 0.1M phosphate buffer, dehydrated and then embedded in an Epoxy Embedding Medium Kit (45349 Sigma-Aldrich, Switzerland). Ultrathin sections (60-nm) were cut with an ultramicrotome, (LKB-8800 Ultratome III Stockholm, Sweden) collected on 400-mesh copper grids,
counterstained with 2% uranyl acetate and then with Sato’s lead. Specimens were observed by a Hitachi H-300 Transmission Electron Microscope.

**Figure 6. Schematic representation of culture process.** Tissue samples from patients with parathyroid adenoma were minced after capsule removal (Step 1), then treated by digestion solution and mechanical disaggregation (Step 2). After digestion the cells were dispersed and cultured to obtain a “Classic Primary Culture” where oxyphil and chief cells grow together (Step 2A). Cell suspension was filtrated to obtain separated oxyphil and chief cells (Step 3A). The big cells or oxyphil cells remain on the filter and grow on it, whereas the small cells or chief cells went through the filter and were cultured on glass slide.
**Phenotypic Characterization of Cells**

To determine whether the isolated cells showed a parathyroid phenotype, we performed real time real time qRT-PCR experiments to measure the gene transcripts Calcium sensing receptor (CaSR), Parathyroid Hormone (PTH), Glial cell missing-2 homolog (GCM2, a specific transcription factor that has been shown to be essential for development of parathyroid gland and is expressed in mature parathyroid) (89-91), Mineralocorticoid Receptor (MR), Glucocorticoid Receptor (GR) and 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2).

Total RNA was extracted from cells using the RNAqueous microkit (Ambion®Life Technologies; Monza, Italy); its integrity and quality was systematically checked with a laboratory-on-chip technology in an Agilent Bioanalyzer 2100 with the RNA6000 Nano assay (Agilent Technologies; Milan, Italy). 1 μg of total RNA were reverse transcribed with Iscript (BioRad; Milan, Italy) in a final volume of 20 μl. The mRNA was measured with a real time qRT-PCR with universal ProbeLibrary probes in the LightCycler 480 Instrument (Roche; Milan Italy). The expression was calculated relative to actin used as an internal control.

**Immunohistochemistry**

For immunocytochemical analysis, oxyphil cells were stained directly on the circular filter (ScreenCell® Cyto) and chief cells on the slide glass. Separated cells were fixed for 10 min with 2% paraformaldehyde in phosphate-buffered saline (PBS) at pH 7.4, and then washed in PBS. Endogen peroxidases were blocked with 0,5% H₂O₂ in PBS for 10 min at room temperature. Samples exposed to anti-rabbit or anti-mouse secondary antibodies were pre-incubated with a permeabilizing buffer (0.2% Tween-20 in PBS) for 60 min at room temperature; samples exposed to anti-sheep antibody (11βHSD2- 11β hydroxysteroid dehydrogenase) were incubated with 0.5% Tween 20 in Super Blocker (ThermoScientific-Rockford, USA) for 60 min at room temperature.
Material and Methods

Separated parathyroid cells were incubated with a monoclonal antibodies at a dilution 1:500 for anti-PTH antibody (GTX39458; GeneTex, Inc. CA. USA), 1:70 for anti-CaSR (ab19347; Abcam Cambridge, UK), 1:50 for anti-Mineralocorticoid Receptor (a kind gift of Dr. Celso Gomez-Sanchez Dept. of Medicine, University of Mississippi Medical Center, Jackson, MS).

Rabbit polyclonal antibodies were diluted 1:300 for anti-Chromogranin A (ab15160 - Abcam - Cambridge, UK), 1:80 for anti-Gcm2 (ab96063 Abcam Cambridge, UK); sheep polyclonal antibody 11βHSD2 (a kind gift of Prof. Paul Stewart Dean of School of Medicine, University of Leeds, Leeds, UK) was diluted 1:200. All primary antibodies were diluted in the same pre-incubation buffer and incubated overnight at 4°C.

After repeated PBS washing, samples were incubated with a secondary antibody and Advance HRP Detection System Kit (Dako Corp. Carpinteria, CA, USA) for mouse and rabbit antibodies, and with secondary antisheep HRP (P0163 Dako Corp, Carpinteria CA, USA) for 11βHSD2 antibody 1:100 for 60 min at room temperature. The immune complexes were visualized with 3,3’-diaminobenzidine (Dako Corp, Carpinteria, CA, USA). Distilled water was used to stop the reaction.

The circular filter and glass coverslip was then dried at room temperature and mounted on a glass slide. Negative controls were carried out by similarly treating sections and omitting the primary antibody. The images were acquired by Leica DMR microscope.

11βHSD2 Protein Expression

Protein expression was quantified by immuno-blot analysis. To this aim APA samples (n=3), normal adrenal gland (n=3), parathyroid gland (n=3), kidney tissue (n=4), A549 cells (Homo sapiens lung carcinoma cells) and HepG2 cells (Human Hepatocyte Carcinoma) were homogenized in 600μl lysis buffer (Thermo Scientific, Italy) using a MagNALyser Instrument (Roche, Italy). Protein concentration was determined in the soluble supernatant with BCA (Thermo Scientific, Italy). Lysate fraction (50 μg) was separated from acrylamide gel and then was electro blotted onto nitrocellulose membrane (Hybond ECL-Amersham Biosciences Europe, Germany). The membranes were blocked for 1h at room temperature in 0,5% Tween 20 in Super Blocker (ThermoScientific-Rockford, USA) and thereafter incubated overnight at 4°C with a
Material and Methods

primary sheep polyclonal antibody against type 2 11βHSD2 (1:100 dilution) (a kind gift of Prof. Paul Stewart Dean of School of Medicine, University of Leeds, Leeds, UK). Blots were analyzed by the QuantityOne Program of VersaDOC 1000 (Bio-Rad, Italy).

**PTH Measurement**

To determine the functionality of parathyroid primary cells we stimulated them with three different concentrations of calcium: 0.5, 1.5 and 3.0 nM for one hour in medium without calcium and low serum concentration (Fetal Bovine Serum 0.5%). Detectable amounts of calcium were detected with an Elisa Assay for Human Bioactive PTH 1-84 (Immutopics, Inc. CA USA). Results were normalized per μg DNA to adjust for variability of cells the number among cultures (92).

**Mitochondrial Membrane Potential**

This was measured based on the accumulation of tetramethylrhodamine methyl ester (TMRM). Cells were seeded onto 24-mm-diameter round glass coverslips and grown for 2-11 days in RPMI-1640 medium 5% FBS (Sigma-Aldrich®, Italy). The extent of cell and, hence, mitochondrial loading with potentiometric probes is affected by the activity of the plasma membrane multidrug resistance pump, which is inhibited by Cyclosporin (Cs) A. Treatment with this drug may therefore cause an increased mitochondrial fluorescence that can be erroneously interpreted as an increase of the mitochondrial membrane potential (93). To prevent this artifact and to normalize the loading conditions, in all experiments with TMRM the medium was supplemented with 1.6 μM CsH, which inhibits the multidrug resistance pump but not the permeability transition pore (PTP) (94). Cells were rinsed once and then incubated in bicarbonate- and phenol red-free Hank’s balanced salt solution (Sigma-Aldrich®, Italy) supplemented with 10mM Hepes and 1.6 μM CsH and loaded with 10 nM TMRM for 30 min. At the end of each experiment, mitochondria were fully depolarized by the addition of 4 μM of the protonophore carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP). Cellular fluorescence images were acquired with an Olympus (Center Valley, PA) IX71/IX51 inverted microscope equipped with a xenon light source (75 W) for epifluorescence illumination and with a 12-bit digital cooled charge-coupled device (CCD) camera.
(Micromax, Princeton Instruments, Trenton, NJ). For detection of fluorescence, 568 ± 25-nm bandpass excitation and 585-nm longpass emission filter settings were used. Images were collected with an exposure time of 100 msec by using a ×40, 1.3 N.A. oil immersion objective (Nikon). Data were acquired and analyzed by using CellR software (Olympus). Clusters of several mitochondria (10–30 clusters) were identified as regions of interest, and fields not containing cells were taken as the background. Sequential digital images were acquired every 2 min, and the average fluorescence intensity of all relevant regions was recorded and stored for subsequent analysis.

**Statistical Analysis**

Results were expressed as mean ± SE of at least 3 separate experiments in which each samples was assayed in duplicated. Differences between groups were analyzed by t-test and, for multiple group comparison, by one-way ANOVA. The differences were considered to be significant at p < 0.05. Statistical analyses were performed using Graphpad/prism 5 for Windows software (Graphpad Software, La Jolla, California, USA; [www.graphpad.com](http://www.graphpad.com)).
Results
RESULTS

Primary Cell Culture from Parathyroid Adenoma

As described in detail in Material and Methods section, cells isolated from parathyroid adenomas were cultured to obtain a classic primary culture where chief and oxyphil cells were grown together. The next step was to separate chief from oxyphil cells and separately grow them in order to obtain two different primary cultures.

All seeded cells were adherent to the bottom of well-plate after 24 hours. In general, more than 95% dispersed cells were viable and grew in columnar aggregates, small clusters or in pseudofollicle formation. We observed that such cell clusters consisted of round cells with different sizes with well defined borders and that they represented the majority of the cultured cells(Fig.7 panel A and B). Occasionally spindle-shaped cells grown far from the clusters, presumably fibroblasts, were found in the culture (Fig.6 panel A). The fibroblast contamination was avoided by immunobinding technique using an antibody against fibroblast membrane marker (CD90) (95). This technique was successfully used in the past by our group with the aim of obtaining a pure population of adrenal cells (96).

Classic primary cultures were passaged once (when the confluence exceed 90% of 6 well plates) but in general the culture was continuous (without passages) because this type of cells need cell-to-cell contacts to survive along the culture (97). Cultures of chief and oxyphil cells were maintained only as continuous culture due to small number of chief cells and the frailty of the oxyphil cells. Since the oxyphil cells showed a poor survival when detached from filters, the cells were cultured on the filters thereby avoiding any cell damage.
Ultrastructure of Parathyroid Adenoma Cells

Cells derived from classic primary cultures were maintained on the plate where they were growing (~5-10x10^5 cells/24 well plates) for the analysis at TEM. Two cell types, different for the amount of mitochondria, were easily distinguishable: one type, presumably oxyphil cells, was very rich in mitochondria that engulfed the cytoplasm, whereas the other cell type, presumably the chief cells, contained mitochondria usually localized only in one portion of the cell. Both cell types were joined by tight junctions thereby forming a continuous layer. Both oxyphil and chief cells contained many vacuoles, glycogen deposits and lipid droplets in the cytoplasm (Fig. 8). Occasionally nuclear pleomorphism was detected.
When the cells were isolated using a filter size-based technique, oxyphil and chief cells were separately cultured and then analyzed in 24-multiwell insert system (~$5 \times 10^4$ cells/24 well plates). Oxyphil cells showed more abundant mitochondria than the chief cells, thereby confirming that the cells rich in mitochondria that we observed in the primary cultures were actually oxyphil cells. Golgi apparatus and endoplasmic reticulum, rough and smooth, were less prominent in both cell types. As observed in the classic primary cultures, both cell types showed secretory granules and large amounts of glycogen (Fig.9).
Figure 8. Ultrastructure of parathyroid adenoma cells. A. Presence of tight junctions (black arrows) original magnification $\times 10000$. B. Glycogen deposits (*) original magnification $\times 7000$. C and D. Mitochondria content: HC Cells with abundant mitochondria, LC Cells with few mitochondria, original magnification $\times 7000$. (C) Cell (N) Nucleus (M) Mitochondria (V) Lipid droplet.
Results

Figure 9. Ultrastructure of parathyroid adenoma cells A. Oxyphil cell with bigger amount of mitochondria and glycogen deposits (*) Original magnification x 5000 B. Adjacent oxyphil cells with abundance of mitochondria. Cellular junctions between them are clearly evident (black arrows). Original magnification x 10000. C. Chief cells with smaller content of mitochondria and glycogen deposits Original magnification x 4000 D. Chief cell. at greater magnification, x 15000 (N) Nucleus (M) Mitochondria.
**Parathyroid Cell Markers: Gene and Protein Expression**

Real-time qRT-PCR showed the expression of parathyroid markers including CaSR, GCM2 and PTH, and the steroids-related markers MR, GR, and 11βHSD2 in the primary classic cultures. No significant differences between primary cell cultures and tissue parathyroid samples collected from same patients with an adenoma at day 5 of culture were found. Likely this was because of the spread values and therefore to the intrinsic cell variability between adenomas (Fig. 10 panel A).

By contrast, the gene expression of most parathyroid cell markers, i.e. CaSR, MR, 11βHSD2 and the transcription factor GCM2, significantly decreased after day 10 as compared to the adenoma tissue (Fig. 10 panel B). A reduction in CaSR expression has been previously described in parathyroid adenomas and hyperplastic parathyroid tissues (10-13), but no information was available for the other markers.

The expression of PTH found in the classic primary culture with real time qRT-PCR was confirmed by immunocytochemistry. The specific immuno-signal for PTH was in fact observed in at least 90% cells, albeit, with differences between cellular types. As previously reported in the literature, PTH was more expressed in the chief than in the oxyphil cells (2). Such difference was found in both classic primary cultures and 'separate' cultures of cell types (observational data) (Fig.11 panel A and Fig.12).

A similar protein expression pattern was found for CaSR and CGA as shown in Fig.11, panels B and C. In contrast, no difference between cell types could be appreciated for the parathyroid specific transcription factor GCM2, which has been held an essential factor for parathyroid development and therefore relevant for both cell types (15) (Fig.11 panel D and Fig.12 panels C and D).
When investigating the steroids-related markers, we found that both classic primary cultures and 'separate' cultured cells express MR and 11βHSD2 proteins (Fig. 13 panels A and B). MR was found to be expressed in the cytoplasm but not in the nucleus in the oxyphil cells, whereas it was expressed in both compartments in the chief cells (Fig. 13 panels C and D). The specificity of the immune-signals was confirmed by the negative controls for each reaction.

The expression of 11βHSD2 in the parathyroid was confirmed by immunoblot experiments. Of interest, the levels of 11βHSD2 protein in the parathyroid tissue were similar to those of adrenal gland or APA, which express at 11βHSD2 to high extent. The levels found in the parathyroid tissue were smaller, even though not significantly, than those found in the kidney, the organ with the highest 11βHSD2 synthesis (Fig. 14).
Results

Figure 10. Parathyroid and steroids-related markers gene expression. A. The expression of most cellular markers in classic primary cell cultures at day 5 does not show significant difference as compared to parathyroid adenoma tissue. Only expression of CaSR significantly decreased (p<0.05) B. Classic primary cell cultures at day 10 showed a significant decrease in CaSR, GCM2 and MR gene expression (n=7)
Results

Figure 11. Immunocytochemistry of parathyroid cell markers in classic culture. A. PTH, Original magnification x 63 B. Chromogranin A, Original magnification x 40 C. CaSR, Original magnification x 40 D. GCM2 staining, Original magnification x 63 (NC) Negative control.
Figure 12. Immunocytochemistry of separated cells. A. PTH staining in oxyphil cells, original magnification x 63 B. PTH staining in chief cells, original magnification x 40 C. GCM2 staining in oxyphil cells, original magnification x 63 D. GCM2 staining in chief cells, original magnification x
Results

Figure 13. Immunocytochemistry of primary cultured cells. A. MR staining in classic primary cultures, original magnification x 63  
B. 11βHSD2 staining in classic primary cultures, original magnification x 40  
C. MR staining in oxyphil cells, original magnification x 40  
D. MR staining in some chief cells  
E. 11βHSD2 staining in oxyphil cells, original magnification x 63  
F. 11βHSD2 staining in chief cells, original magnification x 63. (NC) Negative control.
Results

Figure 14. 11βHSD2 expression in different tissues. Protein expression in parathyroid (lane 2), normal adrenal tissue (lane 3) and aldosterone producing adenoma (APA) (lane 4) was found to be similar to that of kidney tissue (lane 1). A549 and HepG2 cell lines (lanes 5 and 6) were used as negative controls and GAPDH used as internal control.
PTH Production

The ability of the parathyroid cells to produce PTH in the classic primary cultures was investigated at days 5 and 10 of culture by adding Ca\(^{2+}\) at three different concentrations. In presence of low Ca\(^{2+}\) levels (0.5 mM) for 1 hour the production of PTH was significantly increased at days 5 and 10 (\(p=0.028\) and \(p=0.018\), respectively) as compared to the control. No significant effect was observed after 1.25nM and 3nM Ca\(^{2+}\) addition at the same times (\(p=0.68\) and \(p=0.62\), \(p=0.96\) and \(p=0.063\) respectively) (fig. 15).

Mitochondrial Membrane Potential

In normal aerobic conditions the combination of proton pumping by the respiratory chain and of low permeability of the inner mitochondrial membrane (IMM) to charged species allows the build up of a high proton motive force that drives ADP and Pi uptake, and synthesis of ATP by the F1F0ATP synthase (98). The proton motive force has two components: one due to the concentration difference of protons across the membrane (\(\Delta p\text{H}\)) and one ,the membrane potential (\(\Delta \Psi\)) due to the difference in electrical potential between the two aqueous phases separated by membrane. \(\Delta p\text{H}\) in mammals cells is about 0.5-1 units, which correspond to 30-60 mV and \(\Delta \Psi\) is in the order of 140-170 mV (inside negative). The earliest consequence of depolarization (e.g. following impaired respiration) is a switch of the mitochondrial F1F0ATP synthase from its physiological mode of operation to that of an ATP hydrolase, when the glycolytic ATP is available.

Since we found in the TEM experiments that oxyphil cells have higher mitochondrial content than chief cells, we tested whether this can reflects a functional difference in parathyroid primary cells. We therefore monitored the mitochondrial \(\Delta \Psi\) (\(\Delta \Psi\text{m}\)) in oxyphil and chief cells in situ by measuring their accumulation of tetramethylrhodamine methyl ester (TMRM). Cells where treated with CsH, which inhibits the multidrug resistance pump and therefore normalizes cytosolic loading with TMRM, which is a substrate of the pump and could therefore be extruded at rates that vary widely in different cell types. Mitochondria readily accumulated TMRM, indicating that they are energized, and they showed (Fig. 16 panel A) a heterogeneous staining pattern with a
higher fluorescence in oxyphil cells. Addition of the F1FOATP synthase inhibitor oligomycin to the cultures from human adenoma parathyroid cells was followed by increased probe uptake (i.e., by the expected hyperpolarization), followed by a process of TMRM release that appeared to have an earlier onset in oxyphil cells (Fig. 16 panel B). In both cell types fast TMRM release followed addition of the protonophore carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP).
Results

**Figure 15.** Effect of Ca^{2+} on classic primary cultures at day 5 (A) and day 10 (B). Significant increase of PTH was found at low calcium concentration (0.5nM) vs. control (no Ca^{2+} addition).
Results

Figure 16. Changes in mitochondrial TMRM fluorescence induced by oligomycin in primary cell cultures from parathyroid glands. Cell culture established from biopsy was loaded with TMRM and studied as described in "Matherial and methods". A. Representative examples of cells loaded with TMRM before oligomycin addition; oxyphil cells are marked in blue and chief cells in green. B. Where indicated by arrows 6 μM oligomycin (Oligo) and 4 μM FCCP (F) were added. Each trace reports the response of one individual cell.
DISCUSSION

The aim of the present study was to develop a human parathyroid cell line that could be used for investigating the effects of aldosterone on PTH synthesis and secretion. The detection of the MR in parathyroid cells, alongside recent evidences implicating PTH in overproduction of aldosterone in primary aldosteronism in spite of suppression of the renin-angiotensin system, suggests a relationship between parathyroid and the adrenocortical zona glomerulosa. However, the investigation of such relationship has been hampered because of lacking of parathyroid cell line suitable for in vitro studies. Moreover, cultures previously developed in other laboratories do not allow distinction between cell types (99). Here, we describe a novel technique to isolate the two main parathyroid cell subtypes e.g. chief and oxyphil cells.

Primary Cell Culture from Parathyroid Adenoma

Our initial approach was to develop a protocol to obtain a pure primary culture of human parathyroid cells. After several experiments we found that the most advantageous conditions include: (1) automated tissue dissociation to obtain a single-cell suspension, (2) immunobinding-based technique to remove fibroblasts, (3) high cellular confluence (≈3-5x10^5 cells/well) on culture well to stimulate a tissue-like architecture, (4) cell culture medium with moderate serum concentration (5% of total medium volume) and (5) continuous culturing without subculture.

We also found that the protocols that were previously developed in other laboratories did not allow maintenance of functionally active parathyroid cells for long times, most of them being characterized by a rapid and significant loss of PTH secretion within 3-4 days (99-101).

In contrast, using the above described approach we were able to obtain an appreciable PTH production until day 10 of culture. At this time our cultured cells lost CaSR expression. The significance of CaSR expression loss after some days of culture, albeit described by other investigators, is not clear. Reduced CaSR mRNA and protein levels were found in cultured bovine parathyroid cells placed in primary monolayer culture,
Discussion

and this change was associated with reduced responsiveness to Ca\(^{2+}\)(102-108). In contrast, our cells maintained PTH production even after 5 days of culture, likely because the cellular growth occurred in a tissue-like architecture manner. Actually, the short time exposure to collagenase type II (maximum 20 minutes) and the automated dissociation provided a single-cell suspension with no cell damage. Hence, integrity of cell membranes allowed the development of a tissue-like structure, with pseudofollicles at high cellular confluence (~5x10^5 cells/well), which involves reconstruction of extracellular matrix in the plate well. Cultured cells mimicked the original parathyroid structure in the continuous culture, at least for a short period (~10 days).

The contention that organ-like structure is of utmost relevance is supported by data by Ridgeway et al. and Ritter et al. who, some years ago, found that bovine and human parathyroid cells cultured as multicellular aggregates named Organoids maintained their morphology and function, including their response to Ca\(^{2+}\) for at least 2 weeks in vitro. Ritter et al. also were able to reproduce pseudogland in continuous cultures by using collagen support (97,105,109). We tried to exploit the previously described protocols, but unfortunately we could not obtain good results, likely because our tissue samples were very small.

**Parathyroid Cell Morphology, Markers Expression and Function.**

Both intercellular contacts between cells from the same cluster and parathyroid tissue-like architecture play an important role in the maintenance of differentiated cellular function in cultured parathyroid cells. Our primary cells formed clusters up to 10 days (Fig.7), thereby showing cell integrity and ability to resemble the original tissue structure.

By exploiting a novel procedure, we also successfully separated chief from oxyphil cells. The cells were viable expressed specific markers of parathyroid glands, and, more importantly, they were functionally active because were able to produce PTH at least for 10 days, as classic primary cell culture, and expressed CaSR, PTH, GCM2 and CGA. Of interest we first identified steroid-related markers such as MR, GR, and 11βHSD2 in parathyroid cells. Expression of 11βHSD2 in the parathyroid tissue confirmed that steroid-related markers are specific of this tissue. These findings provide experimental
support to the hypothesis that hyperaldosteronism may be caused by primary hyperparathyroidism and vice versa. Further studies are required to better clarify this relationship.

By analyzing fixed tissue samples, two studies reported that key genes of parathyroid function are expressed in both oxyphil and chief cells of hyperplastic parathyroid glands from patients with secondary hyperparathyroidism (2). This finding was in contrast to the old argument that oxyphil cells are 'deactivated' chief cells not expressing parathyroid markers. By showing that both cell types express parathyroid markers, as well a different amount in mitochondria content—a hallmark of parathyroid cell subtypes (110), our study confirm the hypothesis that both oxyphil and chief cells are 'differentiated' parathyroid cells (Fig. 8 and Fig.9).

**Mitochondrial Membrane Potential**

We found by TEM that in parathyroid primary cells cultures oxyphil cells have a higher mitochondrial content respect to chief cell, conserving the pattern already reported in the human parathyroid gland (2). Studying the mitochondrial function by measuring $\Delta \Psi_m$ we observed a heterogeneous pattern in the signal intensity in oxyphil and chief cells (Fig. 16 panel A). Addition of the F1F0ATP synthase inhibitor oligomycin caused hyperpolarization, as expected of phosphorylating cells. Indeed, in respiring cells the $\Delta \Psi_m$ is maintained by proton pumping through the respiratory chain, and ATP synthesis draws a fraction of the proton gradient, which therefore increases when the ATP synthase is blocked. The initial hyperpolarization induced by oligomycin was followed by depolarization in a process whose onset appears to be much earlier in the oxyphil cells. This anomalous response to oligomycin (i.e., depolarization) was also detected in some myopathies, including Ullrich congenital muscular dystrophy (UCMD) and Duchenne muscular dystrophy (DMD) (111,112), where it was attributed to opening of the permeability transition pore (PTP, a high conductance inner membrane channel) following addition of oligomycin. Future studies with CsA, a cyclophilin inhibitor that desensitizes the PTP, will address the question of whether in cells from parathyroid primary human adenoma inappropriate opening of the PTP is also involved in the oligomycin-induced mitochondrial dysfunction observed in these preliminary
Discussion

experiments; and whether the earlier onset seen in oxyphil cells has a pathophysiological relevance. Oxyphil cells are increased in the parathyroid glands of CKD patients, and they have been reported to serve a different, albeit undefined, function relative to chief cells (2,113): additional studies are required to clarify the role of these cells in parathyroid pathophysiology.

Limitations of the Study

We would like to point out that this study has some limitations. One limitation is that we have not yet investigated PTH synthesis in separated cells. This was because of small number of chief cells observed after filtration and poor survival rate of oxyphil cells after detachment from the filter.

Another limitation is the relatively short time of our cultures. Since the parathyroid cells cease to proliferate within few days in culture (108), longer times were not feasible. A further limitation is the small sample size. Therefore supplementary studies are required to clarify the role of cellular subtypes in parathyroid and their possible influence in another target tissues, e.g. adrenal gland.

Clinical Relevance

The findings of our study showing a link between hyperaldosteronism and PTH oversecretion that could have clinical implications not only for primary aldosteronism patients, but also for patients with hyperaldosteronism secondary to the activation of the renin–angiotensin system, as those with heart failure, renovascular hypertension. The occurrence of steroids-related markers found in parathyroid tissue and culture parathyroid cells, as well as the observation that removal of a PTH secreting adenoma was followed by a decrease in blood pressure values (26) support our contention.
**Perspectives**

The observation that oxyphil, but not chief cells, are increased in hyperparathyroidism suggest that the two cell types may play a different secretory and sensing activities. Further studies would be necessary to clarify if the oxyphil and chief cells may activate different signalling pathways in response to secretagogues, e.g. Ca\textsuperscript{2+} or feasibly steroids, and moreover if they undergo apoptosis or hyperplasia when are abnormally stimulated with excess of PTH and/or aldosterone.
ACKNOWLEDGMENTS

The authors wish to thank Professor Celso Gomez-Sanchez from Dept. of Medicine, University of Mississippi Medical Center, Jackson, MS and Professor Paul Stewart Dean of School of Medicine, University of Leeds, UK for the generous gift of the MR and human 11-HSD2 antibodies respectively.

We gratefully acknowledge the contributions, constant support and advice of Professor Maurizio Iacobone (Department of Surgery, Oncology and Gastroenterology), Professor Paolo Bernardi and Alessandra Zulian (Department of Biomedical Sciences) Professor Anna Sandra Belloni and Lucia Petrelli (Human Anatomy Department of Molecular Medicine).

Sources of Funding

This study was supported by research grants by the Young Research Program of the Italy’s Health Minister to L.L. Project GR-2009-1524351, the International PhD Program in Arterial Hypertension and Vascular Biology of the University of Padua, and by FORICA Foundation for Advanced Research In Hypertension and Cardiovascular Diseases.
BIBLIOGRAPHY


