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Expression of Parathyroid-Specific Genes in Vascular Endothelial Progenitors of Normal and Tumoral Parathyroid Glands

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Parathyroid tissue is able to spontaneously induce angiogenesis, proliferate, and secrete parathyroid hormone when autotransplanted in patients undergoing total parathyroidectomy. Angiogenesis is also involved in parathyroid tumorigenesis. Here we investigated the anatomical and molecular relationship between endothelial and parathyroid cells within human parathyroid glands. Immunohistochemistry for CD34 antigen identified two subpopulations in normal and tumoral parathyroid glands: one constituted by cells lining small vessels that displayed endothelial antigens (factor VIII, isolectin, laminin, CD146) and the other constituted of single cells scattered throughout the parenchyma that did not express endothelial markers. These parathyroid-derived $CD34^+$ cells were negative for the hematopoietic and mesenchymal markers CD45, Thy-1/CD90, CD105, and CD117/c-kit; however, a subset of $CD34^+$ cells co-expressed the parathyroid specific genes glial cell missing B, parathyroid hormone, and calcium sensing receptor. When cultured, these cells released significant amount of parathyroid hormone. Parathyroid-derived $CD34^+$ cells, but not $CD34^-$ cells, proliferated slowly and differentiated into mature endothelial cells. $CD34^+$ cells from parathyroid tumors differed from those derived from normal parathyroid glands as: 1) they were more abundant and mainly scattered throughout the parenchyma; 2) they rarely co-expressed CD146; and 3) a fraction co-expressed nestin. In conclusion, we identified cells expressing endothelial and parathyroid markers in human adult parathyroid glands. These parathyroid/endothelial cells were more abundant and less committed in parathyroid tumors compared with normal glands, showing features of endothelial progenitors, which suggests that they might be involved in parathyroid tumorigenesis. ***(Am J Pathol 2009, 175:1200–1207; DOI: 10.2353/ajpath.2009.080979)***

The parathyroid gland is an endocrine organ that dynamically adjusts parathyroid hormone (PTH) secretion in response to changes in extracellular calcium concentrations, thus providing a strict control of ion homeostasis. Although the cellular constitution of the mature gland appears stable under basal conditions, with an estimated steady cell turnover of about 5%/year,[1] dramatic enlargement of the gland size may occur in several pathological conditions. Another peculiar characteristic of parathyroid cells is the ability of spontaneously inducing angiogenesis in both *in vitro* and *in vivo* models. Accordingly, small fragments of parathyroid tissue implanted in the forearm muscle are able to proliferate and to secrete adequate amounts of PTH because the transplanted parathyroid tissue spontaneously contributes to neoangiogenesis.

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Table 1. Clinical and Biochemical Features of the Primary Hyperparathyroid Patients Whose Parathyroid Tumors were Analyzed

No.	Age (yrs)	Sex	Histology	Gland	Cells	Serum Ca^{2+} (mg/dl)	Ionized Ca^{2+} (mmol/L)	Serum P (mg/dl)	Serum PTH (pg/ml)
1	53	F	AD	Left Sup	C	11.7	1.62	2.1	114
2	59	F	AD	Left Inf	C	10.3	1.39	1.9	286
3	70	F	AD	Right inf	C/O	11.9	1.42	2.1	118
4	58	F	AD	Left sup	C	11.5	1.51	1.9	117
5	62	F	AD	Left inf	C	11.2	1.44	2.5	107
6	59	F	AD	Right sup	C	13.2	1.73	2.4	500
7	67	F	AD	Right inf	C	11.1	1.61	2.4	113
8	72	F	AD	Left sup	C	11.7	1.53	2.2	408
9	46	F	AD	Right sup	O	11.1	1.51	2.4	118
10	39	M	AD	Right sup	O	12.2	1.57	2.1	127
11	31	M	AD	Right sup	C	10.6	1.38	1.7	138
12	57	M	AD	Left inf	C	12.7	1.78	2.3	265
13	52	F	HY	Multiple	C	12.2	1.71	2.4	242
14	71	M	HY	Multiple	C	11.9	1.64	2.3	175
15	72	F	HY	Multiple	C	12.7	1.54	1.7	165
16	75	F	HY	Multiple	C	11.8	1.55	1.8	1103
17	82	F	HY	Multiple	C	12.3	1.68	2.5	399

F = female; M = male; AD = adenoma; HY = hyperplasia; Sup = superior; Inf = inferior; C = chief cells; O = Oxifil cells; Ca^{2+} = calcium; P = phosphorus.

Normal values: serum total calcium 8.4 to 10.4 mg/dl; ionized calcium 1.13 to 1.29 mmol/L; serum phosphorus 2.7 to 4.9 mg/dl; serum PTH 10 to 65 pg/ml.

Angiogenesis occurs also in parathyroid proliferative lesions, where it has been demonstrated to be increased compared with normal glands.[2,3] However, secretory activity and tumor size have been found to be either related or unrelated to parathyroid angiogenesis.[2,3] Angiogenesis in parathyroid glands has been studied by evaluating the expression of the specific vascular endothelial marker CD34. Indeed, anti-CD34 antibodies stain hematopoietic cells, as well as mature, immature, and progenitor endothelial cells.[4,5] A positive immunostaining for CD34 antigen have been shown also in stem cell populations from human adult kidney and liver.[6,7]

In the present study, the subpopulation of parathyroid-derived $CD34^+$ cells was isolated and characterized in normal and tumoral parathyroid glands. Unexpectedly, a small proportion of parathyroid-derived $CD34^+$ cells co-expressed both endothelial progenitors and parathyroid specific genes. The parathyroid/endothelial cells showed a different phenotype in parathyroid tumors compared with normal parathyroid, suggesting their involvement in parathyroid tumorigenesis. Finally, parathyroid-derived $CD34^+$ cells displayed some properties suggestive for potential progenitors.

Materials and Methods

Parathyroid Tissues

The study included nine normal parathyroid glands biopsies and 17 parathyroid tumors (five hyperplasia and 12 adenomas) from patients with primary hyperparathyroidism. Patients with the following conditions were excluded: familial hyperparathyroidism, hyperparathyroidism secondary to renal failure, solid or hematological malignancies, or heart failure. Clinical and biochemical data were shown in Table 1. Tissues removed were in part placed in sterile medium for cell culture, in part frozen in liquid nitrogen-cooled isopentane and in part snap frozen in liquid nitrogen and stored at −80°C until analysis. The study was approved by the local ethical committee and informed consent was obtained from all patients.

Immunohistochemistry

For immunohistochemistry, frozen samples were cryostat sectioned. Specimens were fixed, incubated with primary antibodies against CD34 (ready to use, Novocastra, Newcastle on Tyne, UK), von Willebrand factor VIII (vWVIII) (1:100 dilution, Dako spa, Milan, Italy), laminin (1:100 dilution, Sigma-Aldrich S.r.l., Milan, Italy), human endothelial cells (CD146) Alexa Fluor 488 conjugated (1:50 dilution, Chemicon, Temecula, CA), fluorescein lycopersicon esculentum (Tomato) lectin (1:200 dilution, Vector Laboratories, Burlingame, CA), α-smooth muscle actin-fluorescein isothiocyanate-conjugated (1:200 dilution, Sigma-Aldrich), NG2 chondroitin sulfate proteoglycan (1:50 dilution, Chemicon, Temecula, CA), nestin (1:50 dilution, BD Transduction Laboratories, San Jose, CA), CD31 (1:50 dilution, BD Transduction Laboratories, San Jose, CA), ve-cadherin (1:50 dilution, BD Transduction Laboratories, San Jose, CA), PTH (1:50 dilution, BD Biosciences, San Jose, CA), and calcium sensing receptor (1:50 dilution), as previously described.[8] The sections were rinsed in PBS and incubated with corresponding secondary antibodies Alexa Fluor 488, 594, or 647 (1:200, Molecular Probes, Invitrogen Life Technologies, Grand Island, New York) for 1 hour at room temperature. To visualize the cell nuclei, parathyroid sections were counterstained with 4,6-diamidino-2-phenylindole (Sigma-Aldrich), mounted with a Phosphate-Buffered Salines/glycerol solution and examined under a Leica TCS SP2 confocal microscope. Overnight at 4°C, preabsorptions of the primary antisera against both human PTH and

Table 2. Oligonucleotide Sequences Used in RT-PCR Analysis

Primers name	Primers sequence	Annealing temperature	PCR products
GCMB	5′-TAGAAGAAGAGCCATCAAG-3′ (25) 3′-GAGGTATGTTGCTGAAATGA-5′ (25)	47°C	121 bp
PTH	5′-AGCTACTAACATACCTGAACG-3′ 3′-CTCTCCATCGACTTCAGATG-3′	57°C	204 bp
CD31	5′-GGAACATCCTTGGAAATTGGAAGAG-3′ 5′-GCGGTGCTCCCAAGTAGTCTG-3′	65°C	318 bp
Ve-cad	5′-GGAACGTTTCACTGCAAACACACC-3′ 5′-CAGTACTTGTCATGCACCAGTTTGG-3′	67°C	197 bp
CD146	5′-GGTGAATTAGCCTCAATCCC-3′ 5′-CCCGAAGCTCTAGCCAGG-3′	64°C	222 bp
GAPDH	5′-GCCAAAAGGGTCATCATCTC-3′ 3′-GTAGAGGCAGGGATGATGT-5′	47°C	287 bp

GCMB = glial cell missing B; Ve-cad = vascular endothelial cadherin.

calcium sensing receptor (CaSR) had been performed demonstrating the specificity of the antisera used.

CD34$^+$ Cell Purification from Parathyroid Glands

Tissue samples were mechanically dispersed after 45-minute incubation at 37°C with 1 mg/ml collagenase type IA, type II, and type IV (Sigma-Aldrich), into Dulbecco's modified essential medium (Invitrogen Life Technologies, Grand Island, New York) supplemented with 20% fetal bovine serum. Dispersed cells were plated for 24 hours in 12 noncoated wells. The CD34$^+$ fraction was isolated with microbeads selection using MidiMACS and LS columns (Miltenyi Biotechnology, Calderara di Reno, Italy), as previously described.[9] The CD34$^+$ cells eluted as the positively selected fraction were laid on a second column to obtain CD34$^+$ cells of higher purity. The CD34$^+$ fraction was isolated by cell sorting performed on a Fluorescence-activated cell sorting Vantage flow cytometer. This isolation was performed by positive selection of CD34 expressing cells. After selection, living cells were counted with a Burker's camera using Trypan Blue exclusion and an aliquot of the CD34$^+$ cell fraction was analyzed to assess purity. The median purity of enriched CD34$^+$ cells after fluorescence-activated cell sorting (FACS) analysis was 95% (range, 92% to 97%).

Characterization of CD34$^+$ Cells by FACS Analysis

For five-color flow cytometry, 1.5×10^5 cells were incubated with anti-CD34-allophycocyanin, anti-CD133-phycoerythrin (anti-CD133-PE) (Miltenyi Biotechnology, Calderara di Reno, Italy), anti-CD45-PE-Cy7, anti CD90-fluorescein isothiocyanate (BD Biosciences-Pharmingen, San Diego, CA), anti-CD105-PE (Endoglin) (Serotec, Raleigh, NC), anti-CD117-PE (c-Kit) (BD Biosciences-Pharmingen, San Diego, CA), anti CD146 488 alexa fluor, (Chemicon, Temecula, CA), and anti-7-amino-actinomycin D. The controls were isotype-matched mouse immunoglobulins. After each incubation performed at 4°C for 20 minutes, cells were washed in PBS containing 1% heat-inactivated fetal calf serum and 0.1% sodium azide. The cytometric analyses was performed on a FACS Vantage flow cytometer using Cell Quest software (BD Biosciences-Immunocytometry System, San Diego, CA), as previously described.[8,9]

mRNA Expression by Reverse Transcription-PCR

Total RNA was isolated from CD34$^+$ and CD34$^-$ parathyroid cells using Trizol Reagent (Invitrogen, Life Technologies). Aliquots of total RNA (3 μg) were used to analyze glial cells missing B gene (GCMB), PTH, CD146, CD31, and vascular endothelial-cadherin (ve-cadherin) by means of reverse transcription (RT)-PCR technique (Table 2). Genomic DNA amplification was excluded by performing PCR without the RT reaction and using intron spanning primers. The housekeeping *GAPDH* gene was used as internal control. The PCR product were separated by 2% agarose gel electrophoresis, and the specific bands isolated and sequenced to assure they represented the expected products, using an automated sequencer (PerkinElmer Corp., Norwalk, CT).

Laser Microdissection of the Parathyroid-Derived CD34$^+$ PTH$^+$ Cells

The CD34$^+$ cells were isolated from the parathyroid-derived cells by cell sorting and spotted on slides after centrifugation at 1500 rpm for 10 minutes at room temperature by citospin technique. The slides were stained by immunohistochemistry using the primary antibodies mouse anti-human CD34 (Novocastra) and rabbit anti-human PTH (BD Biosciences). The slides were treated with H_2O_2 0.3% in methanol and pre-incubated in horse serum 1%. The primary antibodies were incubated for 1 hour at room temperature. The slides were incubated for 30 minutes with the secondary antibodies (anti-mouse and anti-rabbit respectively) biotin-conjugated and developed by diaminobenzidine. The cells CD34$^+$ and PTH$^+$ were isolated using a laser microdissection system Leica AS LMD (Leica Microsystems, Germany). Using a 63x objective, settings were as follow: aperture 9, intensity 30, speed 5, and offset 22. The areas cut were transferred by gravity into a 0.5 ml tube cap placed

directly underneath the slide. The tube cap was filled with the TRIzol Reagent (Invitrogen) to guarantee the isolation of intact RNA. Total RNA was isolated in according with the manufacturer's protocol. RNA was reverse transcribed as previously described.

In Vitro *PTH Determination*

Both $CD34^+$ and $CD34^-$ cells were cultured in Dulbecco's Modified Eagle's medium/Ham-F10 (ionized calcium concentration 0.3 mmol/L) supplemented with 10% heat inactivated fetal calf serum for 72 hours. Cells were then washed with PBS and incubated with Ham-F10 (containing 0.3 mmol/L Ca^{2+}) for 3 hours. At the end of the incubation, medium was removed and stored at −20°C for determination of human intact 1-84 PTH, using an immunoradiometric assay (Nichols Institute Diagnostic, San Juan Capistrano, CA). The intra- and interassay coefficients of variations were less than 5.7% and 6.7%, respectively, and the sensitivity was 2 pg/ml.

$CD34^+$ Cell Culture and Differentiation

$CD34^+$ cells (5×10^4) were placed on 48-well plates coated with 100 μg/ml fibronectin (Sigma-Aldrich), and incubated at 37°C in a humidified environment with 5% CO_2. After 3 days cells were collected, counted, analyzed by FACS analysis and replated at a density of 2000 cells/cm^2 in DME medium/Ham-F10 (1:1) (containing 0.3 mmol/L Ca^{2+}) supplemented with 10% heat inactivated fetal calf serum and a defined mixture composed of insulin (25 μg/ml), apo-transferrin (100 μg/ml), progesterone (20 nmol/L), putrescine (60 μmol/L), uridine (10 μmol/L), and sodium selenite (30 nmol/L) (Sigma-Aldrich). The following cytokines were added to the medium: leukemia inhibitor factor (20 ng/ml, Sigma-Aldrich), interleukin-6 (20 ng/ml, Sigma-Aldrich), and stem cell factor (50 ng/ml, Sigma-Aldrich). For the clonal culture of parathyroid/endothelial cells, single-sorted $CD34^+$ cells were cultured in multiwell plates in the absence of feeder cells.

For differentiation into endothelial cells, $CD34^+$ cells were cultured in endothelial growth medium [M199 (Gibco BRL, Invitrogen Life Technologies) supplemented with 20% fetal bovine serum (HyClone Laboratories, Logan, Utah), vascular endothelial growth factor (10 ng/ml, Sigma-Aldrich), basic fibroblast growth factor (5 ng/ml, Sigma-Aldrich), heparin (5 U/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and fungizone (0.25 μg/ml)], as previously described.[9] These cells were placed on 48-well plates coated with 0.2% gelatin and were incubated at 37°C in a humidified environment with 5% CO_2.

Statistics

Data are expressed as mean ± SE. When comparing the data from two populations, paired 2-tailed Student's *t*-test or two-ways analysis of variance were used to determine levels of significance. Statistical differences between populations were detected by *Chi-square* test. A *P* value of ≤0.05 was considered statistically significant.

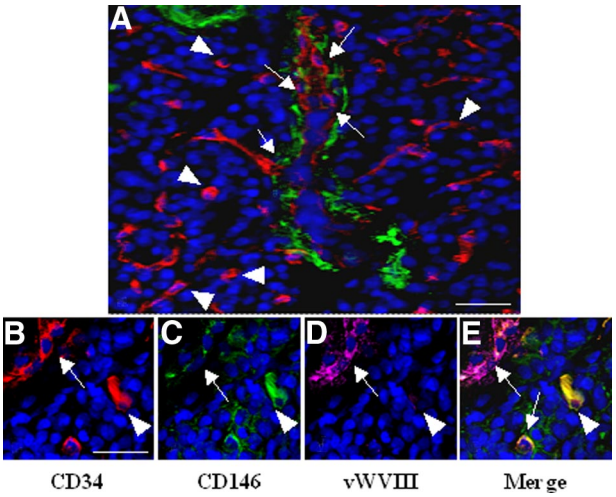

Figure 1. Merge images of immunofluorescence microscopy of tumoral parathyroid section with antibody against CD34 (red) and α-smooth muscle actin (green) (**A**). Nuclei are stained in blue with 4,6-diamidino-2-phenylindole. CD34 immunostaining does not colocalize with α-SMA immunostaining. **Arrows** indicate CD34 cells lining small vessels. **Arrowheads** indicate CD34+ cells scattered throughout the parenchyma. In **B–D**, CD34 (red), CD146 (green), and vWVIII (violet) immunofluorescence staining in a parathyroid adenoma section is shown. Merge image is shown in (**E**). **Arrows** indicate CD34+ cells lining a small vessel co-expressing vWVIII. **Arrowhead** indicates a representative scattered CD34+ cell negative for vWVIII. Scale bars = 20 μm.

Results

Immunohistochemistry Analysis of $CD34^+$ Cells in Parathyroid Sections

We investigated the expression of the endothelial/hematopoietic marker CD34 in tissue sections of normal and tumoral parathyroid glands by immunohistochemistry. $CD34^+$ cells, which were negative for α-smooth muscle actin, were either localized around small vessels or scattered throughout the parathyroid parenchyma (Figure 1, A and B). Most $CD34^+$ cells around small vessels co-expressed CD146, laminin, isolectin, and vWVIII (Figure 1, A–E), resembling the endothelial cell phenotype.[10] Conversely, the $CD34^+$ cells scattered throughout the parathyroid parenchyma, which were in part positive for CD146, were negative for the other markers of mature endothelium, such as vWVIII (Figure 1). The amount of $CD34^+$ scattered cells was higher in parathyroid adenomas and hyperplasia than in normal tissue. $CD34^-$ cells consisted with the parathyroid chief cells.

Isolation and Characterization of $CD34^+$ Cells from Human Parathyroid Tissues

Dispersed cells derived from parathyroid glands were analyzed by FACS on the basis of the expression of CD34 antigen. The proportion of $CD34^+$ cells from adenomatous and hyperplastic glands ($n = 17$) was twice that from

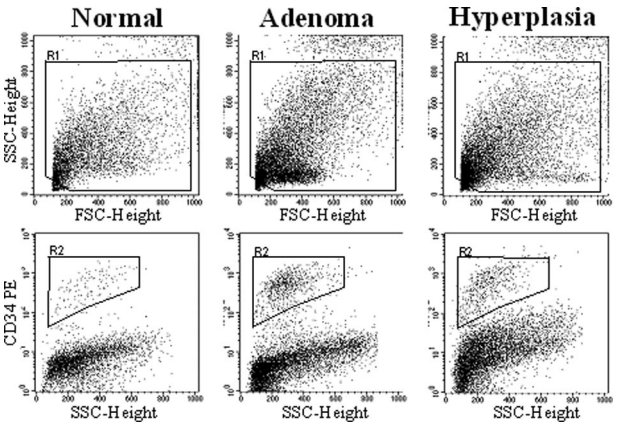

Figure 2. Representative dot plots of parathyroid cell populations, analyzed by flow cytometry. In the **upper panels**, the gates on forward scatter versus side scatter of normal, adenoma, and hyperplasia unfractionated parathyroid cell populations are shown. In the **lower panels**, CD34+ cells derived from parathyroid tissues are shown. The expression of CD34 antigen was evaluated by immunolabeling with PE-labeled anti-CD34. Quadrant marker is defined by PE fluorescence of isotype control. CD34+ cells accounted for 2.2 ± 0.9% of the total normal parathyroid cells (n = 5), for the 4.8 ± 1.9% of the total adenomatous cells (n = 12) and for the 4.2 ± 1.3% of the total hyperplasic cells (n = 5).

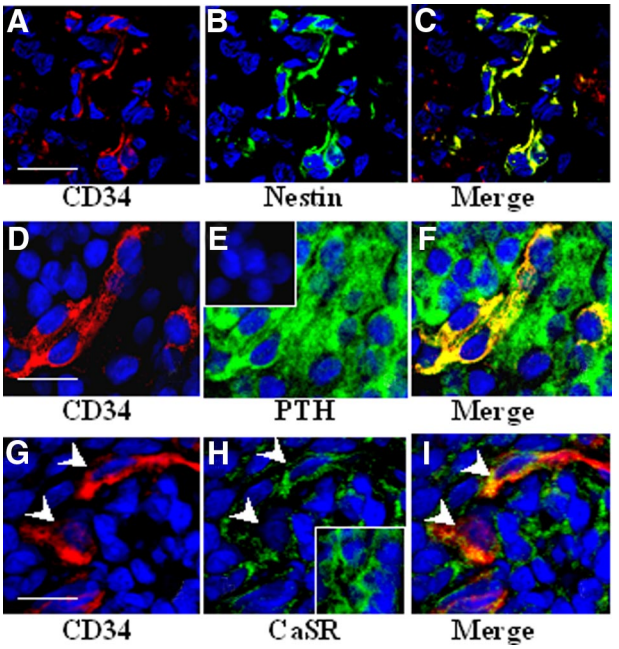

Figure 3. Immunofluorescence microscopy of tumoral parathyroid sections with antibody against CD34 (red) and nestin (green)(**A–C**), PTH (green) (**D–F**), and CaSR (green)(**G–I**). Merge images showing double positive cells in yellow are shown in **C**, **F**, and **I**. **Inset** in **E** shows the immunostaining with preabsorbed anti-human PTH sera. **Inset** in **H** shows the anti-human CaSR staining on a normal parathyroid section. The cross-reactivity of the anti-human PTH antibody with PTH-related peptide was tested on human adult muscle fibers and ruled out. Scale bars = 10 μm.

the normal tissue (n = 5) (4.4 ± 1.2% and 2.2 ± 0.9% of the total cell population, respectively; P = 0.05) (Figure 2). There was no correlation between the percentage of $CD34^+$ cells and age and sex of the patients.

Though the number of $CD34^+$ cells obtained in each experiment limited the number of antigens examined per sample, FACS-based profiling analysis showed that a very low proportion of $CD34^+$ cells expressed the hematopoietic stem markers CD45 (10%), CD133/prominin-1 (0.5–5.5%), and CD117/c-kit (0.1%), suggesting a minor if any contribution of $CD34^+$ cells deriving from bone marrow. Furthermore, $CD34^+$ cells co-expressed neither mesenchymal antigens, such as CD105 and CD90/Thy-1 nor pericyte markers, such as alkaline phosphatase and neural-glial-2 chondroitin sulfate proteoglycan (data not shown). About 50% of $CD34^+/CD133^-/CD45^-/CD90^-$ from normal parathyroid glands co-expressed the pan-endothelial antigen CD146, while this percentage was definitely lower in $CD34^+$ from parathyroid tumors (less than 5%). Moreover, a consistent proportion (about 20%) of $CD34^+$ cells derived from tumoral tissues showed immunopositivity for nestin, a neural stem cell specific marker (Figure 3A–C), while this protein was absent in $CD34^+$ cells derived from the normal gland.

$CD34^+$ Cells Express Parathyroid-Specific Genes and in Vitro *Secrete PTH*

By immunohistochemistry, a small population of $CD34^+$ cells co-expressed PTH protein without any significant difference between normal and tumoral parathyroid tissue (3.8 ± 1.6 vs. 5.1 ± 1.4%)(Figure 3, D–F). $CD34^+$ cells also co-expressed CaSR (Figure 3, G–H), the proportion of CaSR positive cells being reduced in tumoral with respect to normal tissues (5.0 ± 1.4% *vs* 10.0 ± 3.1%, P = 0.03). RT-PCR with oligonucleotides specific for human GCMB and PTH revealed the presence of these two parathyroid specific transcripts in both $CD34^+$ and $CD34^-$ cells sorted from parathyroid (Figure 4A).

To better characterize the expression of PTH from the sorted parathyroid-derived $CD34^+$ cells, a citospin technique was used to spot the sorted cells on slides. Several CD34 and PTH double-positive cells were found after the immunohistochemistry analysis of the spotted cells. A microdissector was used to isolate the $CD34^+PTH^+$ cells. The RT-PCR analysis of the microdissected $CD34^+PTH^+$ cells confirmed the expression of PTH mRNA (Figure 4B).

Immediately after cell sorting, $CD34^+$ cells from both normal (n = 3) and adenomatous (n = 3) glands released a significant amount of PTH in the culture medium (Figure 4B). After 72 hours culture cells obtained from tumoral and normal parathyroid tissues still secreted PTH, the amount of PTH released by tumor-derived, but not by normal-derived, $CD34^+$ cells being significantly higher than that released by $CD34^-$ cells (114 ± 27 vs. 58 ± 21 pg/10^5 cells in 3 hours; P = 0.001) (Figure 4C).

In Vitro *Culture and Endothelial Differentiation of Parathyroid-Derived $CD34^+$ Cells*

The tumoral parathyroid derived $CD34^+$ cells sorted by FACS were cultured in proliferation promoting medium with low calcium concentration (0.3 mmol/L Ca^{2+}). In these conditions, $CD34^+$ cells remained in suspension and maintained a small round shape (Figure 5A), while $CD34^-$ cells were adherent to the support (data not shown). During 30 days culture, the $CD34^+$ cells showed

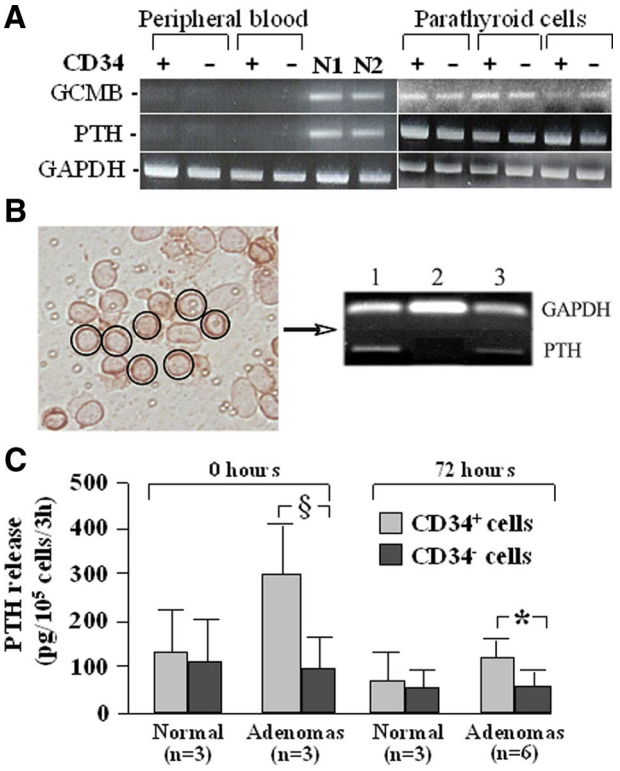

Figure 4. A: RT-PCR analysis for the expression of parathyroid-specific genes mRNA GCMB and PTH. The circulating $CD34^+$ (+) and $CD34^-$ (−) hematopoietic cells sorted from peripheral blood did not express GCMB and PTH mRNAs, while tumoral parathyroid-derived $CD34^+$ (+) as well as $CD34^-$ (−) cells expressed these mRNAs. The lane indicated as N corresponds to the mRNA obtained from a normal parathyroid gland biopsy, used as positive control. mRNA was controlled in each sample by expression of the housekeeping gene *GAPDH*. **B:** Sorted parathyroid-derived $CD34^+$ cells were spotted on slides by cytospin and stained for the expression of CD34 and PTH. Several CD34 and PTH double-positive cells are shown in the **left panel**. In the **right panel** the $CD34^+PTH^+$ cells were isolated using laser capture microdissector and analyzed by RT-PCR for their expression of PTH. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control for the relative quantity of cDNA used in the PCR. Lane 1: positive control; lane 2: negative control; lane 3: CD34/PTH double positive cells. **C:** PTH release from parathyroid derived $CD34^+$ and $CD34^-$ cells immediately after sorting (0 hours) and after 72 hours of culture (72 hours). * $P = 0.001$; § $P = 0.05$.

a doubling time of approximately 72 hours, while the $CD34^-$ cells rapidly (within two passages) became flat cells and entered senescence. However, $CD34^+$ cells could not be expanded with serial passages, suggesting a very low proliferative potential. The low proliferation rate of parathyroid-derived $CD34^+$ cells, which was independent of donor age (ranging from 44 to 82 years), was probably related to the quiescent nature of $CD34^+$ cells. Unfortunately, it was not possible to replicate these experiments in $CD34^+$ cells obtained from normal parathyroid tissue, due to the low number of available cells.

The purified population of parathyroid tumor-derived $CD34^+$ cells showed the capacity to differentiate into endothelial cells when cultured in endothelial medium. Under these conditions, $CD34^+$ cells, but not $CD34^-$ cells, adhered to the support, proliferated with a triangular and refractile morphology (Figure 5B), and after 2 weeks, culture displayed increased expression of CD146 and ve-cadherin mRNA and protein (Figure 5C and data not shown), consistent with differentiation into mature endothelial cells.

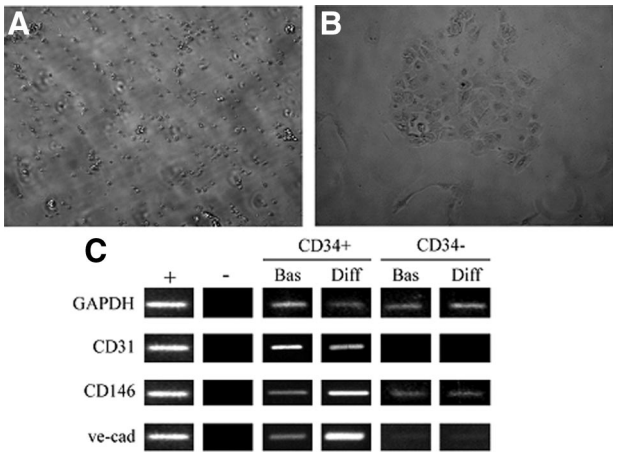

Figure 5. A: Phase-contrast morphology of the parathyroid-derived $CD34^+$ cells sorted by FACS and cultured in the presence of proliferation medium with low calcium concentration (0.3 mmol/L Ca^{2+}). In these conditions, $CD34^+$ cells remained in suspension and maintained a small round shape. **B:** Phase-contrast morphology of $CD34^+$ cells cultured in endothelial differentiating conditions.[9] **C:** RT-PCR analysis of the expression of the endothelial markers CD31, CD146, and ve-cadherin in parathyroid-derived $CD34^+$ (lane 1) and $CD34^-$ cells (lane 2) in basal condition (Bas) and after 2 weeks culture in endothelial conditioned medium (Diff). Human endothelial cell (HUVEC) cDNA was used as positive control (lane +), and C_2C_{12} mouse myoblast cDNA as negative control (lane −); human specific glyceraldehyde-3-phosphate dehydrogenase primers were used as an internal control.

Discussion

The present study first describes a population of cells co-expressing endothelial progenitors and parathyroid-specific genes in the human adult parathyroid. Moreover, immunohistochemistry, FACS, and cell culture data point to the existence of several differences in the molecular and biological characteristics of this cell population in parathyroid tumors, as compared with the normal parathyroid glands. These cells were identified by investigating the expression of the CD34 antigen in normal and tumoral parathyroid. Two previous studies investigated angiogenesis in parathyroid lesions using CD34 staining,[2,3] based on the notion that this cell surface marker binds to vascular endothelium.[11] Both studies reported an increase of $CD34^+$ staining in parathyroid tumors, as compared with normal glands.

Immunohistochemistry analysis confirmed the presence of $CD34^+$ cells in both normal and tumoral parathyroid tissues and provided evidence for the presence of two subpopulations of $CD34^+$ cells which differed for the location and the co-expression of other endothelial markers. One subpopulation was constituted with $CD34^+$ cells lining small vessels, which displayed the cell surface antigen phenotype of differentiated endothelial cells (vWVIII, isolectin, laminin, and CD146), while the other was constituted by single cells scattered throughout the parenchyma, which did not express markers of the differentiated endothelium. Immunophenotyping of $CD34^+$ cells derived from parathyroid glands showed that they

did not display features of bone marrow-derived circulating hematopoietic and mesenchymal progenitor cells.

$CD34^+$ fraction contained a population of cells expressing highly specific parathyroid genes, such as *GCMB*, *PTH* and *CaSR* (5% to 10%). The *GCMB* gene product is an embryonic transcription factor exclusively expressed in parathyroid cells, which is considered to be a master regulator of parathyroid migration, differentiation, and PTH synthesis.[12] Its genetic knock-out is associated with the failure of parathyroid development both in animals and humans.[13–15] Consistent with the notion that GCMB is required for expression of PTH and CaSR in parathyroid/thymus primordium and their maintenance during the subsequent stages of parathyroid differentiation,[16] $CD34^+$ cells expressed these two early parathyroid markers. Moreover, intact 1-84 PTH was released in the medium from $CD34^+$ cells, suggesting the ability of these cells to translate and process PTH into its mature form. This population of $CD34^+$ cells recapitulated the phenotype of the mature parathyroid cell since they also expressed CaSR. Taken together, these data suggest the partial commitment of a population of $CD34^+$ cells to the parathyroid phenotype. This is reminiscent of the fetal liver-derived $CD34^+$ cells that have been reported to express biliary and hepatocellular genes, such as cytokeratin 18 and 19, α-fetoprotein, transferrin, and hepatocyte nuclear factor-4α.[7] More recently, using a similar experimental approach by immunohistochemistry and flow cytometry, Zheng et al documented the presence of cells co-expressing myogenic and endothelial cell markers, included CD34, within the human adult skeletal muscle.[17] Consistent with a progenitor function of this cell population, $CD34^-$ cells in culture rapidly entered senescence, while $CD34^+$ cells proliferated, though at a low rate, and moved through endothelial lineage differentiation, as indicated by their morphology changes and the expression of several endothelial markers.

The $CD34^+$ cell population displayed a number of different features in parathyroid tumors as compared with normal parathyroid glands: 1) parathyroid tumors showed a double percentage of $CD34^+$ cells (about 4% of the total cell population) in comparison with the normal gland by FACS analysis (about 2%); 2) at immunohistochemistry the scattered $CD34^+$ cells were more abundant in tumors compared with the normal glands. Taking into account this localization, it seems unlikely that increased angiogenesis might totally account for the increased number of $CD34^+$ cells in parathyroid tumors[3]; 3) $CD34^+$ cells from parathyroid tumors rarely co-expressed the pan-endothelial antigen CD146; 4) a proportion of $CD34^+$ cells from parathyroid tumors co-expressed nestin, an intermediate filament protein that is considered a marker of neuroepithelial stem cells, also detected in non-neural multipotential stem cells.[18–20] Interestingly, nestin was absent in $CD34^+$ cells from normal parathyroid. These data are consistent with the observation that nestin re-expression in adult tissues occurs mainly under pathological conditions, including injury and neoplasia, possibly associated with angiogenesis[21]; 5) though the proportion of $CD34^+/PTH^+$ cells was similar in normal and tumoral glands, long term cultured $CD34^+$ cells from adenomatous glands released a higher amount of PTH relative to $CD34^-$ cells. This difference was not detected in cells from normal glands; 6) $CD34^+$ cells from parathyroid tumors showed a reduction in the CaSR expression with respect to $CD34^+$ cells from normal glands, in line with the known down-regulation of the CaSR expression in tumoral parathyroid.[22–24] We are tempted to speculate that $CD34^+$ cells might be involved in the angiogenesis supporting the tumorigenesis process and also in the uncontrolled increase in PTH secretion from tumoral parathyroid cells. Indeed, in a murine model of parathyroid tumorigenesis, the insensitivity to extracellular calcium linked to the CaSR down-regulation arises later after the tumoral cell proliferation has occurred.[25]

Admittedly, the parathyroid-derived $CD34^+$ cell population was heterogeneous and a number of experimental limitations (low number of $CD34^+$ cells obtained from each experiment, normal parathyroid of difficult availability for ethical reasons, failure in $CD34^+$ cells expansion due to the very low proliferative potential) prevented a more specific characterization of the single subpopulations. However, the very low proportion of $CD34^+/CD146^+$ cells and the finding of $CD34^+/nestin^+$ cells in parathyroid tumors suggested the presence of a higher proportion of progenitor endothelial cells in tumor as compared with normal parathyroid glands.

In conclusion, we showed that human adult parathyroid contains a population of cells that co-express endothelial progenitors and parathyroid specific cell markers. These parathyroid/endothelial cells were more abundant, less committed, and more functionally active in parathyroid tumors with respect to normal glands. The *in vitro* long-term survival and the endothelial differentiation resemble features suggestive of progenitor function, which indicates the need for further investigations. Although the role of these parathyroid/endothelial cells in parathyroid tumor development still remains undefined, the present study provides a new direction in research for physiology, regeneration, and tumorigenesis of the parathyroid.

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