CLINICAL STUDY

Clinical relevance of thyroid dysfunction in human haematopoiesis: biochemical and molecular studies

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Abstract

Objective: Abnormalities in haematological parameters have been noted in patients with thyroid diseases. Nevertheless, the exact mechanism of thyroid hormones' (THs) action on human haematopoiesis is still not entirely clear.

Design: The influence of THs through TH receptors (TR α -1 and TR β -1) on haematopoiesis in patients with hypo- and hyperthyroidism was analysed.

Methods: TR gene expression at the mRNA and protein levels in human CD34⁺-enriched haematopoietic progenitor cells (HPCs) obtained from the peripheral blood of patients with thyroid disorders and healthy volunteers was analysed. The cell populations were also investigated for clonogenic growth of granulocyte macrophage-colony forming units and erythrocyte-burst forming units (BFU-E). The level of apoptosis was determined by annexin V/propidium iodide staining and quantitative RT-PCR.

Results: The studies revealed that hypo- and hyperthyroidism modify TR gene expression in HPCs *in vivo*. TH deficiency resulted in a decrease in total blood counts and clonogenic potential of BFU-E. In contrast, hyperthyroid patients presented increased clonogenic growth and BFU-E number and significantly higher expressions of cell cycle-regulating genes such as those for PCNA and cyclin D1. Finally, an increase in the frequency of apoptotic CD34⁺-enriched HPCs in hypo- and hyperthyroidism with a modulation of apoptosis-related genes was detected.

Conclusions: The following conclusions were derived: i) TR expression in human haematopoietic cells depends on TH status, ii) both hypo- and hyperthyroidism significantly influence clonogenicity and induce apoptosis in CD34⁺-enriched HPCs and iii) the molecular mechanism by which THs influence haematopoiesis might provide a basis for designing novel therapeutic interventions in thyroid diseases.

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Introduction

Thyroid hormones (THs) play an important physiological role in humans. THs may regulate human haematopoiesis in the bone marrow. The association of thyroid disorders and abnormalities in haematological parameters is well known. In 1881, Charcot showed for the first time that Graves' disease is associated with anaemia. Two years later, Kocher observed a decreased number of red blood cells (RBCs) in the peripheral blood (PB) of patients after thyroidectomy. Hypothyroidism can cause certain forms of anaemia on the one hand or hyperproliferation of immature erythroid progenitors on the other hand. The anaemia is usually macrocytic hypochromic anaemia of moderate severity (1). In contrast, anaemia is not frequently observed in patients

with hyperthyroidism, whereas erythrocytosis is fairly common (2, 3). It has been found that all haematological parameters return to normal when an euthyroid state is achieved (4). As far as white blood cells and thrombocytes are concerned, a slightly depressed total leucocyte count, neutropaenia and thrombocytopaenia have been observed in hypothyroid patients (5). Furthermore, elevated, normal or slightly depressed total leucocyte counts have been found in hyperthyroid patients, with only a relative decrease in the number of neutrophils and a relative increase in the number of eosinophils and mononuclear cells (MNCs). Nevertheless, hyperplasia of all myeloid cell lines in hyperthyroidism and their hypoplasia in hypothyroidism were reported by Axelrod (6). With regard to lymphocytes, triiodothyronine (T₃) has been shown to

be a prerequisite for normal B cell production in the bone marrow through its regulation of pro-B cell proliferation (7, 8). These observations confirmed the association between thyroid gland dysfunction and haematopoiesis. Previously published studies suggested that there is an essential relationship between the hypothyroid state and low levels of iron, vitamin B₁₂ and folic acid in the human body (1, 9). Furthermore, it has been postulated that the influence of THs on haematopoiesis involves an increased production of erythropoietin or haematopoietic factors by non-erythroid cells (10, 11). However, a growing number of studies have demonstrated a direct role of THs in normal human and animal erythropoiesis (12–16).

Based on these data and our previous observations performed *in vitro* that non-physiological concentrations of T₃ directly affect haematopoiesis (17), here we investigated the influence of THs on human haematopoiesis in patients with hypo- and hyperthyroidism. Our studies provide insights into the regulation of haematopoiesis by THs.

Materials and methods

Subjects

We enrolled 50 patients with diagnosed thyroid disease from the Department of Endocrinology and Metabolic Diseases of Pomeranian Medical University in Szczecin, Poland, and a control group of 22 healthy volunteers matched for sex and age. The clinical characteristics of the study population are presented in Table 1. The patients were classified into two groups according to the diagnosis. In the group with hyperthyroidism (25 subjects), there were 13 subjects with Graves' disease and 12 subjects with a history of a toxic nodular goitre. In the group with hypothyroidism (25 subjects), all the patients had a history of Hashimoto's thyroiditis. All procedures were approved by the local ethics committee. Moreover, informed consent was given in every case.

Laboratory measurements

PB samples (15 ml) were collected at the moment of diagnosis and after three months of pharmacological therapy with methimazole for the hyperthyroid patients and levothyroxine replacement therapy for the hypothyroid patients. The samples were collected into EDTA tubes for haematological and hormonal parameters and into heparinized tubes for the isolation of CD34⁺-enriched haematopoietic progenitor cells (HPCs). The haematological parameters white blood cells (WBC), RBC, haematocrit (HCT), haemoglobin (HGB), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular HGB concentration (MCHC) and RDW were evaluated using

Table 1 Clinical characteristics of the study population.

Epidemiological and clinical characteristics	Group 1 (patients with hypothyroidism) <i>n</i>	Controls (healthy volunteers) <i>n</i>	Group 2 (patients with hyperthyroidism) <i>n</i>
Number of subjects/group	25	22	25
Gender (female/male)	20/5	16/6	22/3
	Mean ± s.d.	Mean ± s.d.	Mean ± s.d.
Age (years)	56.79 ± 10.70	46.5 ± 3.04	49.5 ± 14.70
TSH (μIU/ml)	77.02 ± 47.30	2.14 ± 1.30	0.013 ± 0.02
FT ₄ (ng/dl)	0.46 ± 0.29	1.09 ± 0.55	6.373 ± 8.49
FT ₃ (pg/ml)	0.877 ± 0.24	2.71 ± 1.14	6.95 ± 3.13
Disease at diagnosis	%	%	%
Hashimoto's thyroiditis	100.00	0.00	0.00
Graves' disease	0.00	0.00	52.00
Toxic multinodular goitre disease	0.00	0.00	48.00

an automatic blood cell analyser (CELL DYN 3000, Abbott Diagnostics). Unbound free T₃ (FT₃), free thyroxine (FT₄) and TSH levels were measured in the serum using MEIA (Microparticle Enzyme Immuno Assay, Abbott Laboratories, Abbott Park, IL, USA). The reference range for FT₃ was 2.05–3.65 pg/ml; for FT₄, 0.71–1.85 ng/dl; and for TSH, 0.47–5.01 mIU/l.

Cell isolation

The normal light-density MNC fraction was depleted of adherent cells and T lymphocytes (A⁺T⁺ MNCs) as described (18). The isolated fraction was enriched in CD34⁺ cells using a CD34⁺ isolation MiniMACS kit (Miltenyi Biotec, Sunnyvale, CA, USA) according to the manufacturer's protocol. The purity of the collected CD34⁺ cells was over 97%. The viability of cells was assayed by the trypan blue exclusion test as described (19). The cells were counted using a haemocytometer.

RNA isolation and gene expression analysis

Total mRNA was isolated from PB MNCs with the RNeasy Mini Kit (Qiagen GmbH). The mRNA was then treated with DNase I (Gibco BRL) before RT with Moloney murine leukaemia virus RT (Fermentas International Inc., Burlington, Canada). Quantitative assessment of TH receptors (*TRα-1*, *TRβ-1*), *BCL-xL*, *BCL-2*, *BAX*, *Cyclin D1* and *PCNA* mRNA levels was performed by real-time quantitative RT-PCR (qRT-PCR) with an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA).

Primers were designed with Primer Express software (Applied Biosystems). The 25- μ l reaction mixture contained 12.5 μ l of SYBR Green PCR Master Mix, 10 ng of cDNA template and one pair of the primers 5'-GCT GCA GGC TGT GCT GCT A-3' (forward) and 5'-CGA TCA TGC GGA GGT CAG T-3' (reverse) for *TR α -1*, 5'-GTG TCT CAA GTG CCC AGA CCT T-3' (forward) and 5'-CAC AGA GCT CGT CCT TGT CTA AGT AA-3' (reverse) for *TR β -1*, 5'-TCC CTC AGC GCT TGC TTT AC-3' (forward) and 5'-CGC ACA GCA GCA GTT TGG-3' (reverse) for *BCL-xL*, 5'-GCC GGT TCA GGT ACT CAG TCA T-3' (forward) and 5'-CAT GTG TGT GGA GAG CGT CAA-3' (reverse) for *BCL-2*, 5'-GTT GCG GTC AGA AAA CAT GTC-3' (forward) and 5'-GCC GCC GTG GAC ACA-3' (reverse) for *BAX*, 5'-CGG TGT AGA TGC ACA GCT TCT C-3' (forward) and 5'-GCA TGT TCG TGG CCT CTA AGA T-3' (reverse) for *Cyclin D1*, 5'-TCC GCC ACC ATG TTC GA-3' (forward) and 5'-TAT CCC AGC AGG CCT CGT T-3' (reverse) for *PCNA* and 5'-AAT GCG GCA TCT TCA AAC CT-3' (forward) and 5'-TGA CTT TGT CAC AGC CCA AGA TA-3' (reverse) for *BMG*. The threshold cycle (C_t), i.e. the cycle number at which the amount of the amplified gene of interest reached a fixed threshold, was subsequently determined. Relative quantification of *TR α -1*, *TR β -1*, *BCL-xL*, *BCL-2*, *BAX*, *Cyclin D1* and *PCNA* mRNA expression was performed by the comparative C_t method. The relative quantization value of the target, normalized to an endogenous control *BMG* (housekeeping) gene and relative to a calibrator, is expressed as $2^{\Delta C_t}$, where $\Delta C_t = (C_t \text{ of endogenous control gene (BMG)}) - (C_t \text{ of the target gene (TR}\alpha\text{-1, TR}\beta\text{-1, BCL-xL, BCL-2, BAX, Cyclin D1 or PCNA)})$.

To avoid the possibility of amplifying the contaminating DNA, several precautions were taken. First, all primers for qRT-PCR were designed with an intron sequence inside the cDNA to be amplified. Secondly, reactions were performed with appropriate negative controls (template-free controls). Thirdly, uniform amplification of the products was checked by analysing their melting curves (dissociation graphs). Fourthly, the melting temperature was 57–60 °C and the probe melting temperature was at least 10 °C higher than the primer melting temperature. Finally, gel electrophoresis was performed to confirm the correct size of the amplification and the absence of non-specific bands.

TH stimulation of PB-derived CD34⁺ cells

CD34⁺ progenitor cells collected from healthy subjects were incubated for 72 h with an increased concentration of T_3 (Sigma) or with physiological saline without the hormone at 37 °C, 95% humidity and 5% CO₂ under serum-free conditions. Afterwards, the cells were harvested and processed for RNA isolation. The concentration of T_3 used was ten times higher (T_3 10 $\times$ N) than the physiological concentration (N), 3.7 pg/ml.

Protein measurements and western blot analysis

Western blot analyses were performed using extracts prepared from 1×10^6 PB-derived CD34⁺-enriched HPCs to detect the expressions of the TRs. The cells were lysed for 10 min on ice in M-pre-lysis buffer (Pierce Biotechnology, Rockford, IL, USA) containing protease inhibitors (Sigma). Total protein was measured using the Coomassie blue dye method (BioRad protein assay; BioRad Laboratories) with BSA as the standard. The extracted proteins were then separated by 10% SDS-PAGE, and the fractioned proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA). TR α -1 protein was detected using rabbit polyclonal IgG (clone: FL-408, reference: sc-772; Santa Cruz Biotech, Santa Cruz, CA, USA) with HRP-conjugated bovine anti-rabbit IgG as the secondary antibody. TR β -1 protein was detected using mouse monoclonal IgG-1 (clone: J51, reference: sc-737; Santa Cruz Biotech) with HRP-conjugated goat anti-mouse IgG-1 as the secondary antibody. The membranes were developed with an ECL reagent (Amersham Life Sciences) and were subsequently exposed to a film (HyperFilm; Amersham Life Sciences). Each protein sample was analysed in triplicate.

Immunocytofluorescence staining of PB-derived CD34⁺ cells

The CD34⁺ cell population was sorted from PB cells from healthy donors and patients with thyroid disease according to the protocol mentioned above. Cell staining was performed immediately. Briefly, isolated cells were fixed in fresh 4% paraformaldehyde for 20 min, shortly permeabilized by 0.1% Triton X-100 and washed in PBS. TR α -1 protein was detected using rabbit polyclonal IgG (clone: FL-408, reference: sc-772; Santa Cruz Biotech) with ImmunoPure Texas Red conjugated donkey anti-rabbit IgG (Pierce Biotechnology) as a secondary antibody. Cells were subsequently labelled with ready-to-use DAPI solution (BD Biosciences, San Diego, CA, USA) for nuclear staining. For fluorescence images, the BD Biosciences Pathway HT bioimager with a spinning disk-based confocal excitation system (BD Biosciences) was used.

Cell cultures

The CD34⁺ cells (2×10^4) obtained from the PB of the healthy donors and hypo- and hyperthyroid patients were resuspended in 0.4 ml of Iscove's Modified Dulbecco's Medium and mixed with 1.8 ml of the methylcellulose medium MethoCult HCC-4230 (StemCell Technologies Inc., Vancouver, BC, Canada) supplemented with L-glutamine and antibiotics. The appropriate recombinant human growth factors were added to the mixture (20). The growth factors employed for granulocyte-macrophage colony-forming units (CFU-GM) were interleukin-3

(IL-3; 20 U/ml), stem cell factor (SCF) (10 ng/ml) and granulocyte macrophage-colony stimulating factor (GM-CSF) (5 ng/ml) (R&D Systems, USA). Erythropoietin (EPO) (5 U/ml), SCF (10 ng/ml) and IL-3 (20 U/ml) (R&D Systems) were used for erythrocyte burst-forming units (BFU-E). The colonies were counted using an inverted microscope on day 11 in the case of BFU-E and on day 14 in the case of CFU-GM. Cultures developed from each blood sample were performed in quadruplicate. The results are expressed as the percentage of the control value, which is regarded as 100% (18).

Apoptosis assay

The process of apoptosis in the cultures was determined by the combined annexin V and propidium iodide (PI) method (BD Biosciences) following the manufacturer's specifications. The binding of fluorescein-conjugated annexin V and PI was analysed by flow cytometry (FACScan; BD Biosciences).

Statistical analysis

The arithmetic means and s.d.s were calculated on an IBM computer using Statistica version 5.0 software (Chicago, IL, USA). Data are given mostly as the mean $\pm$ s.d., and were analysed using the Mann-Whitney *U* test. Statistical significance was defined as $P < 0.05$.

Results

The characteristics of the patients are summarized in Table 1. In total, 72 subjects were evaluated. The thyroid disease and control groups were matched for age and gender. There were no significant differences in clinical characteristics between the study groups except in thyroid dysfunction type (Table 1).

Changes in haematological parameters of patients with thyroid dysfunction

The blood parameters, i.e. WBC, RBC, HGB, HCT, MCV, MCH, MCHC and RDW, were measured at the moment

of diagnosis and after three months of proper pharmacological treatment. The mean values of the parameters measured in this study are presented in Table 2. Compared with the controls' values, we noticed a significant increase in the number of RBCs in the PB of the patients with hyperthyroidism. We also detected an indicative and significantly decreased level of RBCs in the hypothyroid patients. Similar changes in HGB concentration were recorded for the hyper- and hypothyroid patients respectively. Of note, a statistically significant increase in HCT was observed in the hypo- and hyperthyroid patients. Additionally, the average RBC volume (MCV) was higher in both groups of patients than the reference values, which indicates macrocytosis. However, we observed a well-defined population of 42% of hyperthyroid cases with MCV values below the normal range, which is symptomatic of microcytosis. The MCHC and its average mass (MCH) per RBC were significantly diminished in both the hypothyroid and hyperthyroid patients, indicating a hypochromic state of the RBCs. After three months of standard pharmacological therapy, there were no changes in the standard complete blood counts compared with the results obtained at diagnosis (data not shown). In the group of hypothyroid patients, we could observe TH level normalization after three months of the standard hormone therapy. We observed a normal concentration of T_4 (1.17 ± 0.43 vs 1.09 ± 0.55 ng/dl in healthy subjects). T_3 levels were in a similar range to those of the healthy subjects (3.77 ± 3.24 vs 2.71 ± 1.14 pg/ml respectively). All patients enrolled in the study presented moderate or significant hormonal recovery after T_4 treatment (Fig. 1).

Expression of TRs at the mRNA level

CD34⁺-enriched HPCs isolated from the PB of the patients with thyroid dysfunction and from healthy subjects were used to analyse the expressions of the TRs $TR\alpha-1$ and $TR\beta-1$ using the qRT-PCR method. $TR\alpha-1$ and $TR\beta-1$ transcripts were found in these cells. We found that cells from both groups of patients and the control group expressed mRNA for both the TRs at different levels. $TR\alpha-1$ was found to be mainly

Table 2 Selected blood parameters in patients recruited for the study.

Parameter	Group 1 (patients with hypothyroidism) Mean $\pm$ s.d.	Controls (healthy volunteers) Mean $\pm$ s.d.	Group 2 (patients with hyperthyroidism) Mean $\pm$ s.d.
WBC ($10^3/\text{mm}^3$)	6.6 ± 2.8	7.3 ± 1.2	6.5 ± 1.4
RBC ($10^6/\text{mm}^3$)	$4.17 \pm 0.44^*$	4.58 ± 0.24	$4.85 \pm 0.54^*$
HGB (g/dl)	$12.4 \pm 1.2^*$	13.5 ± 0.7	$14.8 \pm 1.2^*$
HCT (%)	$41.8 \pm 3.4^*$	38.8 ± 2.0	$44.5 \pm 3.7^*$
MCV (μm^3)	$93.2 \pm 4.4^*$	88.5 ± 2.9	$86.2 \pm 5.3^*/93.0 \pm 3.4^*$
MCH (pg)	$26.6 \pm 1.3^*$	30.9 ± 1.6	$28.1 \pm 1.6^\dagger$
MCHC (g/dl)	$32.3 \pm 0.7^\ddagger$	34.8 ± 0.8	$33.5 \pm 1.6^\ddagger$
RDW (%)	13.1 ± 1.2	13.4 ± 0.8	13.6 ± 1.1

* $P < 0.05$; $^\dagger P < 0.01$; $^\ddagger P < 0.001$ versus control values.

Figure 1 Hormonal status of patients after specific hormonal treatment. Each studied subject from the hypothyroid group was independently analysed due to unbound serum free thyroxine (FT₄) concentration before and 3 months after hormonal therapy. The amount of the FT₄ is expressed in ng/dl. Each line represents FT₄ concentration in a different patient.

expressed by CD34⁺-enriched HPCs, with a negligible contribution of *TRβ-1*, which was scarcely expressed (Fig. 2, panel A). Moreover, differences were seen in the expression pattern of *TRα-1* in the groups of hypo- and

Figure 2 Expression of *TRα-1* and *TRβ-1* mRNAs in isolated PB CD34⁺-enriched haematopoietic progenitor cells and changes in *TRα-1* expression depending on thyroid function. (A) Gene expression of *TRα-1* and *TRβ-1* was assessed by real-time RT-PCR in progenitor cells collected from patients enrolled into the study. (B) Gene expression of *TRα-1* in the CD34⁺ cells at diagnosis of hypo- and hyperthyroidism as determined by real-time PCR. The amount of the transcript is expressed in arbitrary units relatively to the control gene BMG ($2^{\Delta C_t}$, where C_t represents the difference in threshold cycle between the control and target gene). Results are expressed as means $\pm$ s.d. of one representative analysis with duplicate of each sample. ** $P < 0.01$, *** $P < 0.001$ versus control group.

hyperthyroid patients. We observed a brisk 50% up-regulation of *TRα-1* in CD34⁺-enriched HPCs from the patients with hypothyroidism ($P < 0.001$; Fig. 2, panel B), whereas the levels of *TRα-1* mRNA tended to decrease by about 37% in the patients with hyperthyroidism and were significantly lower than the basal levels of the control group ($P < 0.01$; Fig. 2, panel B). *TRβ-1* mRNA expression displayed no significant differences among the groups of subjects (data not shown). To confirm or exclude the direct influence of THs on progenitor cells, we employed real-time RT-PCR to quantify the *TRα-1* expression in CD34⁺ cells after 72 h of exposure to highly concentrated T₃. This method provided a chance to assess *TRα-1* expression in cells under two conditions: without contact with T₃ and in a concentration of T₃ ten times higher than normal, which somehow imitates the natural conditions of hyperthyroidism in patients. It was found that *TRα-1* expression was down-regulated after 72-h exposure to highly concentrated T₃ in the majority of the samples from the healthy subjects (Fig. 3). These data confirm the direct influence of T₃ on TR expression. Thus, TRs can regulate the T₃-dependent mechanisms in human HPCs.

Expression of TRs at the protein level

To investigate the molecular changes underlying the enhanced or decreased influence of THs on circulatory stem and progenitor cells, the expressions of *TRα-1* and *TRβ-1* at the protein level were also analysed by western blot. The expression of *TRα-1* was mostly induced in the hypothyroid patients (Fig. 4, panel B); its level was

Figure 3 Expression of *TRα-1* mRNA in isolated PB CD34⁺-enriched haematopoietic progenitor cells and changes in *TRα-1* expression depending on T₃ concentration. Gene expression of *TRα-1* was assessed by real-time RT-PCR in progenitor cells collected from healthy donors and incubated for 72 h with serum saline (control) or with ten times higher than normal concentration of T₃ (T₃ 10 $\times$ N). The amount of the transcript is expressed in arbitrary units. Each line represents *TRα-1* expression in cells collected from a different subject.

Figure 4 Western blot analysis for human TR α -1 and TR β -1 in CD34⁺-enriched haematopoietic progenitors from patients with thyroid dysfunction. Representative western blot analyses of TR α -1 protein levels in the cell extracts collected from hyperthyroid subjects (A) and hypothyroidism group (B) compared to healthy volunteers (C). TR β -1 protein was uniquely expressed in samples from healthy volunteers (D). β -Actin levels are shown as loading controls.

predominantly reduced in the samples from the patients with hyperthyroidism (Fig. 4, panel A). TR β -1 protein was not detected in the patients with hypo- or hyperthyroidism (data not shown). In the control group, we were able to detect both TR α -1 and TR β -1 proteins (Fig. 4, panels C and D). Similarly, immunocytofluorescence of PB-derived CD34⁺ HPCs was used to analyze the expression of TR α -1 protein qualitatively. Figure 5 shows a set of HPCs expressing TR α -1 that were collected from healthy donors (panel B) and from hyperthyroid (panel C) and hypothyroid (panel D) subjects. We could detect marked expression of the thyroid receptors in the cell populations. These data corroborate the results of the western blot analysis of cellular extracts presented above. Of note, patients with hyperthyroidism presented hypercellularity compared with the other groups of subjects.

Clonogenicity of PB-derived CD34⁺-enriched HPCs

To determine whether THs may limit or induce progenitor cell proliferation in the hypo- and hyperthyroid patients compared with the control group, we examined the clonogenic potential of PB-derived CD34⁺-enriched HPCs *in vitro* using proliferation assays. In the hypo- and hyperthyroid patient groups, the number of CD34⁺-expanded CFU-GM colonies was significantly lower than that observed in the healthy euthyroid subjects (36 and 66 vs 100% respectively; $P < 0.05$; Fig. 6). Furthermore, we noted decreased clonogenicity of BFU-E colonies grown from the CD34⁺-enriched HPCs from the hypothyroid patients, and the clonogenic potential was lower by ca. 50% compared with the control cultures. In contrast, erythroid colonies cultivated from progenitors collected from patients with hyperthyroidism exhibited an $\sim 40\%$ increased clonogenic growth compared with the controls ($P < 0.05$; Fig. 7). We also assessed the differences in clonogenicity of CD34⁺-enriched progenitor cells collected from all patients at diagnosis and after three months of the indicated pharmacological therapy and found no significant changes (Figs 6 and 7).

Quantitative determination of cell proliferation

We performed a quantitative analysis of cell proliferation based on the expression levels of mRNA for the

Figure 5 Immunocytofluorescence of PB-derived CD34⁺. Haematopoietic progenitor cells were evaluated for specific expression of TR α -1. Cells obtained from healthy donors (panel B) are compared to cells obtained from patients suffering from hyperthyroidism (panel C) or hypothyroidism (panel D). Nuclei are visualized with DAPI staining in order to show their size and structure (panel A). For panels B, C and D, anti-TR α -1 staining is pseudo-coloured red (Texas Red). Nuclei are pseudo-coloured blue (panels A–D). The expression of each antigen was examined in cells from three independent experiments. Representative and selected data are shown.

Figure 6 Comparative analysis of CFU-GM clonogenicity of human PB CD34⁺-enriched haematopoietic progenitors from healthy volunteers and patients with hypo/hyperthyroidism at diagnosis (dark bars) and after 3-month period of indicated pharmacological therapy (bright bars). The results are expressed as the percentage of the control value taken as 100%. * $P < 0.05$ versus control group.

PCNA and *Cyclin D1* genes, both involved in cell cycle regulation. Samples for gene transcription analysis were collected from the patients with hypo- and hyperthyroidism and were compared with those of the healthy subjects. We observed significant augmentation of the expressions of both the genes in PB-derived CD34⁺-enriched HPCs from the subjects with hyperthyroidism. As shown in Fig. 8, there was a twofold increase in mRNA expression for *Cyclin D1* and a nearly 100% increase in *PCNA* expression in the progenitor cells of these patients ($P < 0.05$ for both). However, mRNA expression for *PCNA* and *Cyclin D1* was not altered in the patients with thyroid insufficiency (data not shown).

Figure 7 Analysis of BFU-E proliferative potential of human PB CD34⁺-enriched haematopoietic progenitors from healthy volunteers and patients with hypo/hyperthyroidism at diagnosis (dark bars) and after 3-month period of indicated pharmacological therapy (bright bars). The results are expressed as the percentage of the control value taken as 100%. * $P < 0.05$ versus control group.

Figure 8 Expression of cell cycle-regulating genes in isolated PB CD34⁺-enriched haematopoietic progenitor cells. *Cyclin D1* mRNA (A) and *PCNA* mRNA (B) were determined by qRT-PCR in the cells from patients with hyperthyroidism (black and white bars) and from healthy controls (black bars). mRNA levels are expressed in arbitrary units as means $\pm$ s.d. of one representative analysis with duplicate of each sample. * $P < 0.05$ versus control group.

Examination of apoptosis of PB-derived CD34⁺-enriched progenitor cells

To assess the indirect influence of THs on apoptosis in haematopoietic cells, two different methods were employed: flow cytometry-related annexin V/PI detection and expression analysis of pro- and anti-apoptotic genes at the mRNA level using qRT-PCR. Combined annexin V/PI staining showed a significant increase in the percentage of cells undergoing apoptosis in the patients with hypo- or hyperthyroidism compared with the euthyroid control subjects ($1723.24 \pm 1259.57\%$ and 1823.43 ± 1334.15 vs 100% of the control cell population respectively; $P < 0.05$). This cellular stain displayed a great percentage of cells especially in the late apoptotic stage (Table 3). To analyze the expression levels of anti- and pro-apoptotic genes in the patients with hypo- and hyperthyroidism, we performed a molecular study of the expression of mRNA for *BCL-2* and *BCL-xL*, both anti-apoptotic genes, as well as for the pro-apoptotic *BAX* gene. Quantitative analysis showed a significantly decreased anti-apoptotic gene expression in

Table 3 Percentage of apoptotic progenitor cells scored with cytometric method in the population of CD34⁺ progenitor cells collected from patients with thyroid dysfunction.

Parameter	Group 1 (patients with hypothyroidism) %	Controls (healthy volunteers) %	Group 2 (patients with hyperthyroidism) %
Progenitor cells in early phase of apoptosis	89.95 ± 114.24	100.00	103.1 ± 74.16
Progenitor cells in the late apoptotic stage	1723.24 ± 1259.57*	100.00	1823.43 ± 1334.15*

Note. Results are expressed as percentage in relation to control values considered as 100%. * $P < 0.05$ versus control values.

both groups of patients. PB-derived CD34⁺-enriched cells harvested from the hypothyroid patients presented only 57 and 74% of the normal expressions of mRNA for *BCL-2* and *BCL-xL* respectively ($P < 0.05$; Fig. 9, panels A and B). Analogously, progenitors collected from the patients with thyroid gland hyperactivity presented decreased expressions of *BCL-2* and *BCL-xL* mRNA compared with the healthy controls (40 and 67 vs 100% respectively; $P < 0.05$; Fig. 9, panels A and B). In the same manner, we assessed the expression of *BAX* gene of pro-apoptotic activity. Here, we also found significant differences between the patients with thyroid disease and the control group. The PB HPCs manifested decreased levels of *BAX* expression by about 24 and 36% respectively in the hypothyroid and hyperthyroid patients compared with the normal *BAX* gene expression level in the healthy volunteers ($P < 0.05$; Fig. 9, panel C).

Discussion

The multiple functions of THs are mediated by the binding of 3,3',5-triiodo-L-thyronine (T₃) to specific nuclear receptors, i.e. TR α and TR β , which are encoded on separate genes. It is well documented that both TRs have two isoforms (21). The concept that THs are directly involved in the haematopoiesis of patients with thyroid disorders prompted us to analyse the influence of THs on human haematopoiesis in patients with hypo- and hyperthyroidism. Our initial studies on human cord blood, PB, and bone marrow CD34⁺-enriched HPCs suggested that T₃ plays a role in the regulation of the growth and apoptosis of human haematopoietic cells (17). Our previously reported investigations demonstrated that exposure to a higher and a lower than normal concentration of TH significantly influenced clonogenicity and induced apoptosis in human HPCs from normal bone marrow, cord and PB (17). However, the action of THs in human haematopoietic progenitors from hypo- and hyperthyroid subjects has not been studied. In the present study, we further elucidated aspects of the influence of THs on haematopoiesis in patients with thyroid disorders *in vivo*.

The expression of TRs at the mRNA and protein levels was previously detected in human PB MNCs (22).

In our previous study, we provided evidence that TRs are present on normal human PB, cord blood and bone marrow CD34⁺-enriched progenitor cells (17). To our knowledge, the present study is the first documented project assessing a PB-derived CD34⁺-enriched HPC population in relation to different thyroid dysfunctions, i.e. hypo- and hyperthyroidism. In the present study, we used qRT-PCR to investigate TR mRNA expression and western blot to detect TR protein expression in haematopoietic progenitors of patients with thyroid disorders. Both groups of patients and the control group expressed mRNA for both the examined TRs at different levels, with a dominating presence of TR α -1. We reported here for the first time that CD34⁺-enriched HPCs from patients with thyroid disorders express TR α -1 at both the mRNA and protein levels. We observed a 50% up-regulation of TR α -1 in the PB-derived progenitor cells of hypothyroid patients ($P < 0.001$), whereas in patients with hyperthyroidism the levels of TR α -1 mRNA were significantly decreased, by around 37% ($P < 0.01$). Additionally, the *in vitro* studies of the direct influence of THs on TR expression in HPCs corroborated the data obtained from our clinical investigation. After 72-h incubation with highly concentrated T₃, the TR α -1 expression in normal CD34⁺ cells tended to be lower in cells exposed to a high dose of T₃ compared with untreated control cells (Fig. 3). These findings may indicate that the abundant presence of THs may reduce the expression of TR α -1 in circulating CD34⁺-enriched HPCs because of intense protein internalization and biodegradation after finishing its role as a receptor binding its ligand. On the other hand, a biologically controlled ligand-negative loop prevents the possibility that more ligand binding to receptors could actively induce different cellular processes. Therefore, the natural reduction of mRNA expression or inhibition of TR production at the protein level results in avoidance of cellular hyperactivity, for example of CD34⁺-enriched haematopoietic stem/progenitor cells, in the hyperthyroid state. In contrast, hypothyroidism induces a high expression level of TR α -1, resulting in an alertness state in the cells that are prepared to bind the scarcely circulating THs to the abundantly expressed TRs. Moreover, TR β -1 expression was hardly detectable at the mRNA level in all the examined groups.

Figure 9 Genes with cell death/survival functions are expressed in PB-derived CD34⁺-enriched haematopoietic progenitor cells and are differentially modulated by thyroid hormones. Changes in the expression of *BCL-2*, *BCL-xL* and *BAX* genes were determined by qRT-PCR method in progenitors from patients with hyperthyroidism (black and white bars) and hypothyroidism (checked bars) and from healthy controls (black bars). mRNA levels are expressed in arbitrary units as means $\pm$ s.d. of one representative analysis with duplicate of each sample. * $P < 0.05$ versus control group.

These observations indicate that the expression of TRs is dependent on TH status. Previous observations by Meier-Heusler that suggested a non-significant trend towards a down-regulation of *TRβ-1* mRNA levels in PB MNCs of hyperthyroid patients support this notion (23). Similarly, Féart *et al.* documented that in hypothyroid subjects, dramatically low T_3 and T_4 concentrations were associated with a decrease in the expression of *TRβ-1* in PB MNCs (24). These data suggest that TR expression in HPCs is sensitive to the pathological conditions related to hypo- and hyperthyroidism.

Furthermore, it has been revealed that the expression of *TRα-1* decreases in older adults by around 13%, and in the case of *TRβ-1*, the expression decreases by 18% (26).

In our study, we also assessed the effect of thyroid status on the clonogenic potential of PB-derived CD34⁺-enriched HPCs. We demonstrated that the clonogenic potential of CD34⁺-expanded CFU-GM is lower in hypothyroid and hyperthyroid patients than in euthyroid subjects. We postulate here that the decreased clonogenic growth of PB-derived CD34⁺-enriched HPCs might be associated with the high percentage of apoptotic cells, which were observed in patients with hypothyroidism or hyperthyroidism, and with significantly reduced expression of anti-apoptotic genes in the HPCs of these patients. Data generated from *in vitro* culture studies correlate with WBC counts obtained from blood samples collected at diagnosis of the patients with thyroid dysfunction. We observed a tendency of reduced amounts of white blood cells in the patients with thyroid disorders compared with the healthy controls.

The influence of TH status on PB-derived BFU-E growth was also assessed. We noted a growth reduction in CD34⁺-expanded BFU-E from hypothyroid patients compared with the euthyroid subjects. This study gives results consistent with our previous findings that showed a negative proliferative effect on BFU-E colony growth after incubation of cord blood-, PB- and bone marrow-derived haematopoietic stem/progenitor cells with T_3 concentrations lower than physiological levels (17). Nevertheless, we found a significant increase in clonogenic potential of CD34⁺-derived BFU-E obtained from hyperthyroid patients compared with their euthyroid controls. This effect might be a result of the modulatory action of THs on the production of EPO. It has been demonstrated that a high concentration of THs increases the production of this hormone (10, 11, 25). However, in our model of *in vitro* cultures with an established amount of EPO given at the moment of growth induction, we observed the increased growth potential only in the samples collected from the hyperthyroid patients. It seems that THs can induce signal transduction pathways involved in haematopoietic stem cell proliferation other than erythropoietin-dependent ones. Furthermore, we observed highly overexpressed genes involved in cell cycle regulation, such as *Cyclin D1* and *PCNA*, in CD34⁺-enriched HPCs from those patients. Especially, the twofold increase in *Cyclin D1* expression might be responsible for the observed significant induction of clonogenic growth of erythrocyte progenitors in our model. This observation suggests that the growth of immature progenitor cells requires a significant contribution of TH that stimulates the observed up-regulation of cell cycle-dependent genes. These data correlate strongly with erythrocyte-related parameters, such as RBC, MCV, MCH and MCHC, from the blood samples collected at diagnosis. We noted a significant increase in the number of RBCs in the PB of

the hyperthyroid patients. These cells were subsequently characterized by increased MCV, which indicates macrocytosis, as well as by a reduced corpuscular HGB concentration and mass. These findings together might indicate that blast forms of erythrocytes that are overproduced in the bone marrow of hyperthyroid patients under the stimulation of TH-dependent cell proliferation circulate in the PB. However, we observed a well-defined population of hyperthyroid cases with MCV values below the normal range, which is symptomatic of microcytic anaemia and might be caused by enteric iron malabsorption (3).

In contrast, we detected significantly decreased levels of RBCs in hypothyroid patients. Similarly, low HGB concentrations were recorded, which clinically indicates anaemia. In the same notion, we determined that these patients presented a hypochromic state of RBCs, as indicated by the significantly diminished values of MCH and MCHC. These data corroborate our findings that CD34⁺-expanded BFU-E from hypothyroid patients reveal a significant growth reduction *in vitro* which might be related to the *in vivo* suppression of TH production in these patients. On the other hand, the findings of other groups and our own data indicate that patients with hypothyroidism due to Hashimoto's thyroiditis suffer from macrocytic anaemia that can be related to autoimmune processes in peripheral tissues (26).

After three months of pharmacological treatment, we did not observe any significant changes in clonogenic growth of patient-derived BFU-E or CFU-GM compared with the results obtained at diagnosis in both groups of patients. However, it is likely that in most cases of hypothyroidism, the disease regresses slowly and the 3-month pharmacological treatment may not be sufficient to affect the proliferative potential of HPCs in these patients.

THs, as many other factors, have been shown to be important regulators of apoptosis via receptor-mediated processes *in vitro* as well as *in vivo* (27, 28). Mihara *et al.* noted a significantly higher percentage of apoptotic T lymphocytes from patients with Graves' disease than from normal donors (29). Unfortunately, the mechanism of pro-apoptotic signal transduction has not been entirely clarified (28). In our study, we demonstrated a significant increase in the percentage of apoptotic CD34⁺ cells in both hypo- and hyperthyroid patients compared with healthy volunteers (Table 3). This finding is in accordance with our previously published data showing that incubation with non-physiological concentrations of T₃ induced programmed cell death in human cord blood-, PB- and bone marrow-derived CD34⁺-enriched HPCs (17).

A strong correlation between BCL-2 down-regulation and spontaneous apoptosis has been reported by various groups (30). We investigated the expressions of anti-apoptotic *BCL-xL* and *BCL-2* genes and pro-apoptotic *BAX* gene at the mRNA level in the

CD34⁺-enriched HPCs. We previously demonstrated that incubation with non-physiological concentrations of T₃ affects the expressions of the pro- and anti-apoptotic genes (17). Here, we determined whether genes with cell death/survival functions are expressed and are differentially modulated by THs in PB-derived CD34⁺-enriched HPCs from patients with thyroid disorders. We established a strong relation between the thyroid status of the patients and the expressions of the anti- and pro-apoptotic genes. Cells harvested from hypothyroid patients presented decreased expressions of mRNA for *BCL-2* and *BCL-xL* of around 43 and 26% respectively. We noted an even more marked decrease in the expressions of both anti-apoptotic genes in the hyperthyroid group, with 60 and 33% diminished levels of *BCL-2* and *BCL-xL* respectively, compared with the healthy controls. All data were of statistical significance. Analogously, progenitors collected from patients with either type of thyroid dysfunction presented a significantly decreased expression of the pro-apoptotic gene *BAX* at the mRNA level. *BAX* is expressed at high levels during the period when programmed cell death normally takes place, and gene down-regulation occurs shortly thereafter. This could markedly decrease the number of *BAX*-expressing cells in the population of CD34⁺-enriched progenitor cells in both groups of patients. However, this mitochondria-associated pathway might not be related to the TH levels of patients, and perhaps other, for example extrinsic, ways of apoptosis induction, for example via FasR, also play a role in the programmed cell death observed in these patients.

In conclusion, we revealed here a direct cause-and-effect association between thyroid disorders and human haematopoiesis. Our study demonstrates for the first time that TRs are sensitive to the pathological conditions present in hypo- and hyperthyroidism regarding their expression. These studies revealed that hypo- and hyperthyroidism modify TR gene expression in haematopoietic progenitors *in vivo*. Our data also indicate that both hypo- and hyperthyroidism significantly affect the proliferative potential of HPCs. Similarly, these data revealed that the action of THs strongly influences the apoptotic process in HPCs by affecting the expressions of pro- and anti-apoptotic genes, such as *BCL-2*, *BCL-xL* and *BAX*, which reflects the very complex nature of this process in humans *in vivo*. Taken together, these data demonstrate that THs modulate cell production in the bone marrow. Our observations may help improve the understanding of the interactions between TH function and human haematopoiesis and provide a basis for designing a novel view for diagnosis and therapeutic intervention in thyroid diseases.

Declaration of interest

All the authors of the work state that no competing financial interests exist.

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