CLINICAL STUDY

Detection of thyroglobulin mRNA transcripts in peripheral blood of individuals with and without thyroid glands: evidence for thyroglobulin expression by blood cells

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Abstract

Objective: Recent studies have assigned clinical significance and prognostic value to the detection of thyroglobulin (Tg) mRNA in the blood of patients subjected to total thyroidectomy for a papillary or follicular thyroid carcinoma. In this study, we investigated the diagnostic specificity of Tg mRNA detection, analysing blood samples from healthy volunteers and from patients previously subjected to total thyroidectomy for reasons other than a carcinoma of the follicular epithelium.

Design and Methods: Total RNA was extracted from whole blood, reverse-transcribed and the cDNA amplified for Tg and glyceraldehyde-3-phosphate dehydrogenase with specific primers. Expression levels were analysed by using a semi-quantitative PCR. In a few cases, Lymphoprep gradients were used to separate the mononuclear and polymorphonuclear cells prior to further analysis by reverse transcription/PCR.

Results: Our data suggested that all individuals expressed Tg mRNA. Moreover, no differences in the expression levels between subjects with and without thyroid glands were documented. Documentation of Tg expression by the mononuclear and polymorphonuclear layers in patients without thyroid glands support the hypothesis that both lymphocytes and granulocytes express Tg and may justify a background expression in blood, independently of the presence of follicular cells in circulation.

Conclusions: Tg mRNA expression is not limited to follicular cells of the thyroid gland, and its expression by normal blood cells should be considered in tests performed for diagnostic purposes.

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Introduction

Clinical interest in serum measurement of thyroglobulin (Tg) in the follow-up of patients with well-differentiated carcinomas of the thyroid gland deriving from follicular cells has been largely documented. However, this method is limited by a low sensitivity (1, 2) and by the interference of anti-Tg antibodies (3, 4).

In an attempt to overcome the above problems and to increase the sensitivity of detection of residual disease, a reverse transcription/PCR (RT-PCR) approach was developed (5, 6) and appears to be valuable and promising method for monitoring such patients.

The detection of Tg transcripts in normal individuals has been documented (6, 7). This can be tentatively explained either by the presence of follicular cells in circulation, as documented by Ringel et al. (6), or by illegitimate transcription of Tg by blood cells. The importance of the latter possibility has been underestimated.

In order to define the contribution of blood cells to the basal expression of Tg mRNA, we studied two groups of individuals: one comprised healthy volunteers, and the other comprised patients previously subjected to total thyroidectomy for reasons other than a thyroid carcinoma deriving from follicular cells.

Materials and methods

Samples

EDTA-blood samples were obtained from two groups of individuals: Group A (n = 10) comprised healthy volunteers; Group B (n = 10) comprised patients subjected to total thyroidectomy for larynx carcinoma (n = 2), for multinodular goitre associated with compression of the trachea and paroxysmal dyspnoea (n = 2), and for medullary thyroid carcinoma (n = 6). Histological samples were re-evaluated to exclude the presence of a papillary micro-carcinoma. Patients in
Group B were undergoing therapy with liothyroxine. Moreover, all the patients in the latter group presented undetectable levels of serum Tg and none had antibodies against Tg.

**RNA extraction**

Total RNA from whole blood was extracted using two commercially available RNA-extraction kits, depending on the quantity of blood available and according to the manufacturer’s recommendations (TRIzol LS, 3 ml whole blood (Life Technologies, Inc., Gaithersburg, MD, USA), or Rneasy Blood Mini Kits, 1.5 ml whole blood (Qiagen, Inc., Valencia, CA, USA).

In a few cases an additional sample was processed in order to separate the mononuclear fraction (lymphocytes + monocytes) from the polymorphonuclear fraction (granulocytes). Approximately 6 ml fresh blood were used for Lymphoprep density-gradient centrifugation according to the instructions of the manufacturer (Nycomed Pharma Diagnostics, Oslo, Norway). Additionally, the layer containing the granulocytes was further processed by osmotic erythrocyte lysis and subsequent washing steps. RNA was extracted from both fractions by using TRizol according to the manufacturer’s instructions.

**RT-PCR**

An aliquot of total RNA (1 μg) was reverse-transcribed with SuperScript (Life Technologies, Inc.) in a 20 μl reaction volume with random primers. Thereafter, 1 μl first-strand cDNA was used as the template for the PCR reaction. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was also determined in all samples to assess the integrity of the RNA. The primers used are shown in Table 1. The cycling conditions for Tg were as follows: initial denaturation at 95 °C for 5 min, followed by a variable number of cycles of denaturation at 94 °C for 45 s, annealing at 59 °C for 45 s, extension at 72 °C for 45 s, and final extension at 72 °C for 7 min. Cycling conditions for GAPDH were the same, except for the annealing temperature, which was 64 °C. The reactions were run on a UNO II thermocycler (Biometra, Göttingen, Germany).

The identity of the PCR product for Tg was confirmed by digestion with NlaIV (New England Biolabs, Inc., Beverly, MA, USA), according to the conditions specified by the manufacturer, as well as by direct sequencing of purified PCR products by using the Sequenase Version 2.0 kit (USB, Cleveland, OH, USA).

**RT-PCR assay sensitivity**

The analytical sensitivity of the assay was assessed by using serial dilutions of total RNA, and the RT-PCR was performed as described above.

**Semi-quantitative PCR**

To compare the levels of Tg expression between the two groups, we adapted a method used by Stevens et al. (9) to compare the levels of corticotrophin-releasing hormone-receptor expression. After determining the linear range of amplification of Tg cDNA, we chose three progressive amplification cycles within this range, i.e. 32, 34 and 36. The linear range of amplification of the housekeeping GAPDH cDNA was also determined, and the amplification cycles used were 20, 22 and 24. Within these ranges, the PCR products showed a linear increase in signal intensity. Each set of samples was electrophoresed on 10% ethidium-bromide-stained polyacrylamide gels. The images were visualized, collected and recorded with Eagle Eye II (Stratagene, La Jolla, CA, USA). The relative intensity of the cDNA was then quantified from negatives by using one-dscan for Windows (Scananalytics, Inc., Billerica, MA, USA). Analysis of Tg expression was achieved by calculating the average ratios of the relative optical densities of Tg to GAPDH at the three cycle numbers for each sample. The mean ratio was then determined for each group.

**Tg measurement**

Serum Tg was measured by using the immunoradiometric assay ELSA-hTG (CIS Bio International, France), the lower detection limit being 0.5 ng/ml.

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**Table 1** Sequences of oligonucleotides used as primers. All primer sets span exon junction sites so that cDNA and genomic DNA amplification products can be distinguished by size.

<table>
<thead>
<tr>
<th></th>
<th>Sequence</th>
<th>Position</th>
<th>Fragment length (bp)</th>
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<tbody>
<tr>
<td>Tg</td>
<td>Sense: 5′-cag cag cag gca att gct tt-3′ (designed by us)</td>
<td>Bases 7288–7307</td>
<td>349</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5′-aat tct gca gtg cct ggt-3′ ref. no. 8</td>
<td>Bases 7619–7636</td>
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<tr>
<td>GAPDH</td>
<td>Sense: 5′-cgt ctc cac cac cat gga gaa-3′ ref. no. 5</td>
<td>Bases 375–395</td>
<td>330</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5′-gag gca ggg atg atg ttc tg-3′ (designed by us)</td>
<td>Bases 685–704</td>
<td></td>
</tr>
</tbody>
</table>
Statistical analysis

Data are expressed as means±s.e.m. Differences were tested by using the Student’s t-test. Significance was defined as the $P < 0.05$ level.

Results

We were able to detect Tg transcripts as the expected 349 bp amplicon in all samples. Tg was expressed not only by healthy individuals with thyroid glands (Fig. 1A), but also by subjects without thyroid glands (Fig. 1B). No transcripts were identified in the water control or in the RT-negative control. In serial dilutions of RNA, the expected band was detected in 10 ng total RNA, and an increase in band intensity was noticed with increasing amounts of RNA (Fig. 2A). GAPDH was detected in as little as 10 pg total RNA (Fig. 2B).

Enzyme digestion with NlaIV and direct sequencing confirmed the identities of the PCR fragments. The restriction analysis is shown in Fig. 3.

The semi-quantitative PCR for Tg mRNA was performed as described in Methods. The progressive Tg cycles (32, 34, 36) as well as the progressive GAPDH cycles (20, 22, 24) are illustrated in Fig. 4. Comparative analysis of semi-quantitative PCR for Tg mRNA between individuals with and without thyroid glands did not reveal any differences (Group A, 0.62±0.06; Group B, 0.78±0.16).

Separate RT-PCR analyses of the mononuclear and polymorphonuclear layers enabled us to demonstrate that both expressed Tg (Fig. 5). Experiments included samples of healthy controls and of individuals without thyroid glands. The results were similar for both groups.

Discussion

Currently, the postoperative follow-up of patients with well-differentiated tumours that originate from the follicular epithelium depends on serum-Tg measurement. The major limitations of this method are the presence of anti-Tg antibodies, which may interfere with the Tg immunoassays, and the interpretation of values obtained in patients undergoing suppressive therapy.

The molecular detection of tissue-specific gene expression in peripheral blood of patients with solid tumours was initially documented for prostate and neuroblastoma malignancies (10, 11). Tg is an iodinated glycoprotein of 660 kDa that consists of two identical subunits. It is secreted by the thyroid follicular cells and encoded by a gene with at least 48 exons (12).
Ditkoff et al. (5) reported, for the first time, a method for detecting Tg mRNA in peripheral blood of patients with well-differentiated thyroid tumours. Since the authors were not able to document Tg expression either in controls or in patients with benign thyroid disease, Tg mRNA appeared to be a specific tumour marker. Subsequently, different studies revealed that Tg transcripts could be found in blood from healthy volunteers (6, 7), in a variety of human cell lines (7), and in different human tissues and organs (13, 14). Such inconsistent results suggested that different RNA-extraction methods, RT-PCR conditions, and primers could interfere with the final results. Particular to our assay is the use of primers to the 3′ extremity (as opposed to primers to the 5′ extremity used in previous studies).

A recent study (13) clearly documented the fact that a change to the number of PCR cycles was sufficient to alter the percentage of positive cases obtained in different groups of individuals with benign and malignant thyroid diseases.

The detection of Tg transcripts in the blood of healthy individuals may correspond to expression by follicular cells in circulation as well as illegitimate expression by blood cells. The latter possibility, although admitted (7, 13), has never been fully addressed.

Using a semi-quantitative approach, we were unable to find any difference in the expression level of Tg when comparing healthy volunteers with subjects without thyroid glands. It can be argued that the presence of small amounts of thyroid tissue is a possibility that cannot be excluded for patients subjected to thyroidectomy, since postoperative thyroid scans were not performed. In spite of this, the two groups are objectively different in terms of thyroid tissue.

Moreover, when blood was processed to separate the mononuclear layer and the polymorphonuclear layer in order to obtain RNA from each layer, we documented Tg expression in both layers. When present in the circulation, follicular cells will be found, after density-gradient centrifugation, in the mononuclear layer. Since we cannot exclude the possibility of circulating follicular cells in healthy control subjects, the present study was designed to include thyroidectomized patients.

All experiments were performed in subjects without leucocytosis or haematological disease. It is therefore unclear as to whether Tg is stably expressed by blood cells or, on the contrary, modulated in response to different clinical conditions (e.g. during infections).

Clinical use of RT-PCR requires standardization of protocols, longitudinal studies, and a quantitative approach (15). It seems likely to be important to define the normal cut-off point of Tg mRNA in blood in order
to make correct interpretations of moderately elevated Tg mRNA values in patients showing no evidence of disease (16). Unless increasing values are observed over time, borderline cases may correspond to a higher background expression, and a diagnosis of recurrence might not be warranted.

Acknowledgements
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References