Intrathyroidal cytokine gene expression profiles in autoimmune thyroiditis

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Abstract

Cytokines are thought to mediate the initiation and perpetuation of autoimmune thyroiditis. However, this concept is mainly based on in vitro findings and to date only interleukin (IL)-6 and interferon-γ (IFN-γ) have been detected in Graves’ disease in vivo. The cytokine pattern produced by T-helper (Th) cells has important regulatory effects on the nature of the immune response. We therefore determined these cytokine mRNAs in Graves’ disease and Hashimoto’s thyroiditis.

RNA was extracted by cesium chloride gradient centrifugation from the thyroid tissue of 12 patients undergoing thyroid resection for Graves’ disease and from two patients being treated for Hashimoto’s thyroiditis. Two patients with parathyroid adenomas and one patient with a goiter were used as controls. RNA was also extracted from normal human thyroid epithelial cells in primary culture. The cDNAs were prepared by reverse transcription and amplified for IL-2, -4, -5, -6 and -10 and IFN-γ by polymerase chain reaction.

All the cytokine mRNAs were detected in the Hashimoto’s thyroid glands in large quantities. Six of the 12 Graves’ disease thyroid glands showed, when compared with controls, an increased accumulation of transcripts for: IFN-γ, IL-2, -4 and -10 or IL-2, -4 and IFN-γ or IL-2 and IFN-γ or IFN-γ alone, each in one case or IL-2 alone in two cases. These cytokine profiles were not representative of a Th1 or Th2 phenotype. Increased amounts of cytokine mRNA in thyroid glands from Graves’ disease patients were mostly associated with high microsomal antibody titres and/or prominent intrathyroidal lymphocytic infiltration. IL-6 and/or IL-10 mRNAs were detectable in all Graves’ disease thyroid glands and in control thyroid tissue. IL-10 mRNA was not detectable in normal human thyroid epithelial cells in primary culture.

Graves’ disease and Hashimoto’s thyroiditis clearly differ with respect to the number of positive intrathyroidal cytokine mRNAs and their levels. The different cytokine patterns in Graves’ disease and in Hashimoto’s thyroiditis could reflect the clinical spectrum of autoimmune thyroiditis which is characterized by thyroid tissue destruction and/or thyroid autoantibody production. These data suggest that the course of autoimmune thyroiditis is regulated by the interplay of several cytokines.

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Introduction

The thyroid gland in autoimmune thyroiditis is predominantly infiltrated by T lymphocytes (Paschke et al. 1992). According to in vitro investigations, cytokines and especially interferon-γ (IFN-γ) are thought to mediate the initiation and perpetuation of autoimmune thyroid disease. However, a plethora of in vitro activities of cytokines and some contradictory findings have been reported. To date, substantial in vivo synthesis has been demonstrated for interleukin (IL)-6 in Graves’, Hashimoto’s and also normal thyroid epithelial cells (Zheng et al. 1991). In spite of the absence of no obvious difference between autoimmune thyroiditis and the non-toxic goiter, the normalisation of increased IL-6 serum concentrations upon remission of subacute thyroiditis has been reported (Bartalena et al. 1993). Immunohistological staining of cryostat sections for IFN-γ was only detected in two out of five Graves’ disease thyroid glands (Margolick et al. 1988), whereas IFN-γ-containing lymphocytes were found in each paraffin-embedded tissue from 31 cases of thyroid autoimmunity and also, although less frequently, in control thyroid tissues (Hamilton et al. 1991). Unexpectedly, in Graves’ disease and Hashimoto’s thyroiditis, protective effects of IFN-γ against cell-mediated cytotoxicity have been observed (Bogner et al. 1988). In spite of several in vitro effects of elevated levels of tumour necrosis factor-α (TNF-α) on thyroid epithelial cells, TNF-α mRNA could not be detected in thyroid tissue from patients with Graves’ disease (Paschke et al. 1993a).

In the light of these findings, the in vivo network of pathophysiological functions of cytokines in autoimmune
Thyroiditis has been difficult to determine. However, the cytokine pattern produced by T-helper (Th) cells has important regulatory effects on the nature of the immune response. Autoimmune thyroiditis comprises a spectrum ranging from Graves’ disease with predominant functional stimulation of the thyroid tissue to Hashimoto’s thyroiditis with a predominance of thyroid tissue destruction. Transitions from Graves’ disease to Hashimoto’s thyroiditis have been described (Hirotu et al. 1986, Tamai et al. 1989). Progressive failure of thyroid function because of destruction of the thyroid tissue is common in long-standing Graves’ disease and has been suggested as one of the possible mechanisms of remission in Graves’ disease (Wood & Ingbär 1979). However, the intensity of the intrathyroidal autoimmune process is not reflected by thyrotrophin-stimulating hormone (TSH) receptor antibody levels (Paschke et al. 1993b) and the individual clinical course of Graves’ disease cannot be predicted by the determination of thyroid antibodies or other parameters (Schluesener et al. 1989). We therefore evaluated the patterns of intrathyroidal cytokine mRNA in Graves’ disease and Hashimoto’s thyroiditis by using a reverse polymerase chain reaction (RT-PCR) in order to investigate the mechanisms of autoimmune thyroid disease and the intrathyroidal expression of cytokines in vivo in autoimmune thyroid disease.

Materials and Methods

Thyroid tissue was obtained from 12 patients undergoing subtotal thyroid resection for relapsing Graves’ disease (patient nos 1–12) and from two patients with Hashimoto’s disease undergoing thyroid resection because of tracheal compression (patient nos 13 and 14). Consent was obtained from each patient after full explanation of the purpose and nature of all the procedures used. The diagnosis of Graves’ disease was based on a diffuse technetium scintiscan uptake, elevated TSH-receptor antibodies and/or endocrine ophthalmopathy and elevated concentrations of thyroid hormones. The diagnosis of Hashimoto’s disease was established by the presence of a goiter, elevated thyroid antibodies and hypothyroidism. Control thyroid tissue was obtained from two patients undergoing surgery for hyperparathyroidism and from one patient undergoing thyroid resection for nodular goiter (patient nos 15–17). Patient nos 1–5 with Graves’ disease received preoperative iodine treatment on a routine basis while none of the other patients did so. Additional clinical data for the patients investigated are listed in Table 1. Thyroid microsomal antibodies were determined with an enzyme immunoassay (Elias, Freiburg, Germany). After switching to the routine determination of thyroid peroxidase antibodies, these were determined with a radioimmunoassay (Henning, Berlin, Germany). TSH-receptor antibodies were detected using a radioligand assay (Henning).

Thyroid tissue was snap frozen in liquid nitrogen and stored at −80°C. Total Graves’, Hashimoto’s and control thyroid tissues were used for mRNA extraction. For IL-10 mRNA detection in cultured thyroid epithelial cells, peripheral thyroid tissue was obtained from patients undergoing thyroid resection for a cold nodule. After collagenase digestion of the thyroid tissue and filtration, the cells were pelleted at 80 g. Thyroid follicles were harvested by washing the pellet twice with basal Eagle’s medium, thereby eliminating cell debris and single cells, including fibroblasts. At the time of harvest, the cells were subconfluent. Total RNA from cultured thyroid epithelial cells and from total thyroid tissue was extracted by cesium chloride gradient centrifugation (Sambrook et al. 1989).

cDNA was prepared with oligo-dT primers and murine myelomonocytic lymphoma virus (MMLV) reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD, USA). Total RNA (1 µg) and oligo dT primers (1 µg) in 10 µl H2O (total volume) were heated to 65°C for 10 min, cooled on ice and subsequently incubated for 1 h at 37°C with 10 µl of a pool containing 6-6 µl 3× concentrated reverse transcription buffer (Tris, 150 mM, pH 8.3; KCl, 225 mM; MgCl2, 9 mM; dithiothreitol 30 mM; 2 µl dNTP, 10 µM; 0.5 µl RNAsin (Promega, Leiden, The Netherlands); and 1 µl MMLV reverse transcriptase (200 U/µl)). The samples were then heated for 5 min at 94°C. Comparison of cDNA preparation with oligo dT primers or random hexamers gave slightly superior results for all cytokine cDNAs amplified with the oligo dT approach. PCR amplification was performed using 5 µl cDNA for cytokines and 1 µl for hypoxantine phosphoribosyl transferase (HPRT) and β-actin (and 4 µl H2O) after initial heat denaturation at 93°C for 2-5 min: 35 cycles (except for IFN-γ; 40 cycles) at 55°C for 2 min, 72°C for 3 min and 93°C for 1 min, followed by 6 min at 72°C in an automated thermocycler (Trio Thermoblock, Biometra, Göttingen, Germany). The total reaction volume was 25 µl containing 2-5 µl of 10× concentrated buffer (Tris, 100 mM, pH 8.3; KCl, 500 mM; MgCl2, 10 mM; and gelatine, 0.01%); 0.5 µl dNTP, 10 µM; 0.5 µl of each primer at a concentration of 1 µg/µl; 0.25 µl Taq DNA polymerase (Boehringer Mannheim, Germany) and 15-75 µl H2O. As a positive control, cDNA was prepared from normal peripheral blood mononuclear cells isolated using a Ficol gradient, incubated in RPMI 1640 (Life Technologies Inc., Gaithersburg, MD, USA) with 10% fetal calf serum and stimulated with lipopolysaccharide (LPS). Amplification of cDNA with primers for the housekeeping genes coding for HPRT or β-actin were used as controls. The primers used for PCR amplification are shown in Fig. 1. These primers amplify cDNA fragments of 265, 317, 291, 628, 578, 359, 500 and 661 base pairs respectively. The sequences of the IL-10 primers were chosen in regions of the human IL-10 gene with no homology with the viral IL-10 (Kim et al. 1992). Sense and antisense primers were complementary to sequences in the
first and last exons respectively, or spanned exon–exon junctions and are therefore mRNA-specific. In addition to their predicted size, the specificity of the amplified bands has previously been validated by restriction enzyme digests giving appropriately sized fragments or by probing with 32P-labelled internal oligonucleotides (Ehlers & Smith 1991, Yamamura et al. 1991). Reverse PCR amplification was repeated at least once for each sample. The PCR amplification products (12.5 μl) were visualized by ethidium bromide staining after separation on a 2% agarose gel with the 1 kb ladder or Puc digested with TaqI and Sau3 (Bethesda Research Laboratories) as molecular weight markers.

Results

PCR amplification results for intrathyroidal cytokines are shown in Fig. 2. The HPRT PCR product was of similar magnitude for all samples, except for a slight difference in sample no. 7. For IL-2, -5, -6 and -10, positive PCR amplification results were obtained in control thyroid tissue. For IFN-γ, 40 instead of 35 PCR cycles had to be used in order to visualize transcripts in the controls. In spite of the apparent sensitivity of the PCR method, which was demonstrated by the detection of cytokines in the controls, IFN-γ was undetectable in several samples. For IL-4, positive results associated with undetectable cytokine mRNA in controls were observed for four samples. Increased IL-10 and IL-2 PCR products over controls could be observed in five and seven samples respectively. IL-5 and -6 PCR products were not increased in any of the samples when compared with controls, except for IL-5 in the Hashimoto samples. Because the amount of RNA available was limited, sample no. 10 was not amplified for IL-6 in this experiment, but gave a positive result in other amplifications.

The two Hashimoto thyroids showed an increased cytokine mRNA signal for IFN-γ and IL-2, -4, -5 and -10, when compared with controls. Six Graves’ thyroids were characterized by an increased level of cytokine mRNA: sample no. 8 for IFN-γ, IL-2, -4 and -10 and a faintly positive band for IL-5; sample no. 12 for IL-2, IL-4 and IFN-γ; sample no. 11 for IL-2 and IFN-γ and samples no. 1 and no. 2 for IL-2. Moreover sample no. 7 showed an increased mRNA signal for IFN-γ mRNA and was the only sample which was negative for IL-10 and -6 mRNA along with a weaker amplification result for HPRT. Clinically, three of the six Graves’ patients with increased cytokine mRNA levels (nos 1, 12 and 8) were characterized by very large lymphocytic infiltrates (++) and two of the six patients (nos 2 and 12) by high thyroid peroxidase or thyroid microsomal antibody titres, whereas one of these six Graves’ disease patients (no. 7) had low thyroid autoantibody titres and hardly any intrathyroidal lymphocytic infiltration. There was no correlation between increased cytokine mRNA levels and preoperatively detectable TSH-receptor antibodies (Table 1).

There was no positive PCR amplification result for IL-10 in cDNA prepared either from unstimulated isolated thyroid epithelial cells or from those stimulated with forskolin for 3 h (Fig. 3).

Discussion

PCR is a semiquantitative technique at best. Furthermore, qualitative interpretations, namely the presence or the absence of a transcript, are dependent upon the conditions of PCR amplification. However, reverse PCR is the more sensitive method for the in vivo detection of cytokines. This is illustrated by the lack of lymphokine mRNA detection in T cell clones from Graves’ and Hashimoto’s thyroids without the use of reverse PCR (Grubeck-Loebenstein et al. 1990). In vitro T cell stimulation did not compensate for this lack of sensitivity since it induced the production of nearly all cytokines in T cell clones from autoimmune thyroiditis as well as from non-toxic goiters which no longer permit any discrimination (Grubeck-Loebenstein et al. 1990). Moreover, T cell stimulation with mitogens does not reflect spontaneous cytokine secretion and must be carried out in vivo. The conditions for PCR amplification of cytokine cDNAs in our study were sensitive enough to detect, even in normal thyroid tissue, all cytokine transcripts except IL-4. This high sensitivity led to the detection of IL-2 mRNA in normal thyroid tissue, although its presence is usually confined to activated lymphocytes. Increased cytokine mRNA levels are suggested when the intensity of the amplified fragment for a given sample is clearly higher than all controls. The PCR technique does not enable us to distinguish which cells are producing the mRNAs.

The cytokine mRNA expression clearly distinguishes the two cases of Hashimoto’s thyroiditis from those with Graves’ disease and from controls. Hashimoto’s thyroiditis
**Figure 2.** Analysis of interferon-γ, interleukins-2, -4, -5, -6, -10 and hypoxanthine phosphorybosyl transferase (HPRT) gene expression using a reverse polymerase chain reaction method. Lanes 1–12 = patient nos 1–12 with Graves' disease; lanes 13 and 14 = patients nos 13 and 14 with Hashimoto's disease; lanes 15–17 = controls nos 15–17; W = negative control; P = positive control; M = molecular weight marker (1 kb ladder, Bethesda Research Laboratories or Puc/TaqI, Sau3).
TABLE 1. Patients clinical data: thyroid-specific medication, TSH-receptor antibodies and thyroid peroxidase or thyroid microsomal antibodies determined prior to surgery

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Diagnosis</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Thyroid-specific medication</th>
<th>TBI (U/l)</th>
<th>TPOAb (U/ml)</th>
<th>Lymphocytic infiltration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Graves' disease</td>
<td>25</td>
<td>F</td>
<td>Carbimazole</td>
<td>28</td>
<td>*&gt;400</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Graves' disease</td>
<td>63</td>
<td>F</td>
<td>Carbimazole</td>
<td>48</td>
<td>*1116</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Graves' disease</td>
<td>28</td>
<td>F</td>
<td>Carbimazole</td>
<td>128</td>
<td>*7415</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Graves' disease</td>
<td>26</td>
<td>M</td>
<td>Carbimazole</td>
<td>0</td>
<td>*&gt;400</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Graves' disease</td>
<td>32</td>
<td>F</td>
<td>Carbimazole</td>
<td>18</td>
<td>*&gt;400</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Graves' disease</td>
<td>41</td>
<td>F</td>
<td>Methimazole, thyroxine</td>
<td>&lt;5</td>
<td>&lt;500</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>Graves' disease</td>
<td>38</td>
<td>M</td>
<td>Methimazole</td>
<td>&lt;5</td>
<td>744</td>
<td>+</td>
</tr>
<tr>
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<td>F</td>
<td>Methimazole</td>
<td>&lt;5</td>
<td>&lt;500</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>Graves' disease</td>
<td>53</td>
<td>M</td>
<td>Carbimazole</td>
<td>&lt;5</td>
<td>&gt;3000</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>Graves' disease</td>
<td>39</td>
<td>M</td>
<td>Methimazole, thyroxine</td>
<td>11</td>
<td>&lt;500</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>Graves' disease</td>
<td>37</td>
<td>M</td>
<td>Methimazole</td>
<td>25</td>
<td>&lt;500</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>Graves' disease</td>
<td>33</td>
<td>F</td>
<td>n.d.</td>
<td>134</td>
<td>&gt;3000</td>
<td>+</td>
</tr>
<tr>
<td>13</td>
<td>Hashimoto's disease</td>
<td>28</td>
<td>F</td>
<td>None</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>14</td>
<td>Hashimoto's disease</td>
<td>57</td>
<td>F</td>
<td>Thyroxine</td>
<td>&lt;5</td>
<td>&lt;500</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td>Hyperparathyroidium</td>
<td>66</td>
<td>F</td>
<td>n.d.</td>
<td>&lt;5</td>
<td>&lt;500</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>Hyperparathyroidium</td>
<td>n.d.</td>
<td>F</td>
<td>n.d.</td>
<td>&lt;5</td>
<td>&lt;500</td>
<td>0</td>
</tr>
<tr>
<td>17</td>
<td>Recurrent goiter</td>
<td>33</td>
<td>F</td>
<td>Thyroxine</td>
<td>n.d.</td>
<td>n.d.</td>
<td>90</td>
</tr>
</tbody>
</table>

Lymphocytic infiltration was graded semiquantitatively by 0 = absence of lymphocytes, + = some lymphocytic infiltration, ++ diffuse lymphocytic infiltration in the scanned area.

n.d. = not determined; TBI = TSH-receptor binding inhibition antibodies, TPOAb = thyroid peroxidase antibodies; MAb = microsomal antibodies. Antibodies values marked with asterisk are TPOAb, those without asterisk are MAb.

is characterized by a major increase of IFN-γ and IL-2, -4 and -10 mRNA and, to a lesser extent, IL-5 mRNA. However, half of the Graves' disease thyroids did not show elevated cytokine levels and, for the positive Graves' disease patients, the level of cytokine mRNA seemed to be much lower compared with the two Hashimoto's thyroids samples. Only six of the 12 Graves' disease thyroid glands showed an increased mRNA level for the six cytokines investigated at this advanced stage of the disease. However, all Graves' disease thyroid glands were positive for IL-10 and/or II-6 mRNA, both of which are also found in normal thyroid tissue.

The intense amplification of IL-6 mRNA for all samples is consistent with the previously demonstrated production of IL-6 by thyroid epithelial cells from Hashimoto's, Graves' and normal thyroid glands (Zheng et al. 1991). Since IL-10 was also detected in most of the thyroid glands and since keratinocytes have been shown to produce IL-10 (Enk & Katz 1992), we investigated the possibility that IL-10 might also be produced by thyroid epithelial cells. However, we could not amplify any IL-10 product from cDNA prepared from cultured, unstimulated and forskolin-stimulated human thyroid epithelial cells (Fig. 3).

The detection of increased IL-2 and IFN-γ mRNA levels in only five and four of the 12 Graves' disease patients respectively is in accordance with undetectable IFN-γ mRNA levels by Northern blot (Schuppert et al. 1993). Furthermore, there was no increase in the number of IL-2-producing T cell clones derived from Graves' infiltrates compared with control lymphoid tissues (Mariotti et al. 1989) and only 80% of T cell clones produced IFN-γ in Graves' disease as compared with 59% in control lymphoid tissue (Mariotti et al. 1989). Moreover, no significant difference for intrathyroidal IFN-γ mRNA detected by quantitative slot blot analysis was found between Graves' disease and non-toxic goiter (Grubeck-Loebenstein et al. 1989). All these low prevalences of IFN-γ have been detected in patients with Graves' disease undergoing subtotal thyroid resection. It is therefore important to know whether the infrequent amplification of IFN-γ and IL-2 signals in the Graves' disease thyroids is limited to this advanced stage of the disease, since especially IFN-γ is thought to play a central role in the initiation of autoimmune thyroiditis (Botazzo et al. 1983).

Our analysis of cytokine gene expression demonstrated increased levels of IL-2, -4, -10 and IFN-γ RNA in all Hashimoto's and some Graves' patients. This pattern does not support the hypothesis that the pathogenesis of autoimmune thyroiditis includes simple Th1 or Th2 immune responses (Mosmann & Moore 1991; Romagnani 1992). Our methodological approach did not allow us to discriminate which cells actually produce these cytokines. The profile of cytokines found in six cases of Graves' disease (nos 1, 2, 7, 8, 11 and 12) could characterize the immune basis for the potential of this disease to go into...
clinical remission via cell-mediated tissue destruction or to continue with chronic TSH-receptor antibody production.

All except one Graves’ disease patients in our study with elevated cytokine patterns were characterized by high microsomal antibody titres and/or very large lymphocytic infiltrates (++, Table 1). Patient no. 7 was the only Graves’ patient with preoperatively undetectable thyroid antibodies and hardly any intrathyroidal lymphocytic infiltration. On the other hand, two patients who were positive only for IL-10 (no. 9) and 6 (nos 3 and 9) were also characterized by high microsomal antibody titres and very large lymphocytic infiltrates (++, Table 1). The available clinical data may therefore be insufficient to correlate cytokine patterns and clinical characteristics unequivocally. Moreover, in certain basal conditions, TNF-α mRNA can be found without detectable TNF-α protein (Beutler et al. 1986). These results illustrate that cytokine mRNA levels do not always correlate with protein production.

These results demonstrate that Graves’ disease and Hashimoto’s thyroiditis are characterized by a complex dysregulation of the cytokine network involving changes of several cytokine mRNA levels. These observations are in agreement with the finding that over-expression of only IL-2 in islet cells of transgenic mice results in inflammation but not autoimmunity (Allison et al. 1992). Furthermore, treatment of patients with chronic hepatitis with recombinant human IFN-γ or IFN-γ was not associated with the appearance of thyroid autoantibodies (Kung et al. 1990). However, induction of autoimmune thyroiditis, as described by the appearance of thyroid peroxidase and thyroglobulin antibodies, was observed in five of 14 patients treated with IL-2 and IFN-α (Pichert et al. 1991). Treatment with IL-2 and LAK cells exacerbated pre-existing autoimmune thyroiditis characterized by microsomal and thyroglobulin antibodies in five out of 34 patients (Atkins et al. 1988). All these findings also suggest that several cytokines are involved in the pathogenesis of...

Figure 3. (a) Reverse polymerase chain reaction (PCR) analysis of interleukin-10 mRNA level using cDNA prepared from isolated thyroid epithelial cells stimulated with forskolin (lane 3) or unstimulated (lane 4). Lane 2, positive control and lane 1, negative control. M=molecular weight markers (1 kb ladder, Bethesda Research Laboratories or Puc/TaqI, Sau3). (b) β-actin PCR amplification from thyroid epithelial cells stimulated with forskolin (lane 1) and unstimulated (lane 2) are shown. M=molecular weight markers.
autoimmune thyroiditis. Further studies are required to elucidate the interplay of these cytokines in the induction and progression of autoimmune thyroiditis.

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