Euthyroid sick syndrome and inhibitory effect of sera on the activity of thyroid 5'-deiodinase in systemic sclerosis

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Abstract

Objective
Our aim was to demonstrate the occurrence of euthyroid sick syndrome in patients with systemic sclerosis (SSc). Furthermore, the presence of anti-thyroid antibodies and their relationship to thyroid 5'-deiodinase activity was investigated.

Methods
The activity of thyroid 5'-deiodinase was measured by 5' outer ring-deiodination using the sera of patients with SSc (n=21), undifferentiated connective tissue disease (n=12), and secondary (n=19) and primary (n=11) Raynaud’s syndrome (RP). Patients with acute cardiovascular events at the time of the study (n=16) were investigated as controls.

Results
Low FT₃ (FT₃<2.5 pg/ml) was frequently demonstrated in all the patient groups (9/21, 3/12, 10/19 and 8/11, respectively). The high frequency of a FT₃/FT₄ ratio <0.2 representing euthyroid sick syndrome was also often found in SSc (14 cases) and primary (12 cases) and secondary (6 cases) RP patients. Anti-thyroid peroxidase antibody was detected in 17 patients with SSc and in 7, 8 and 3 cases with undifferentiated connective tissue disease, secondary and primary Raynaud’s phenomenon, respectively, and in none of the controls. The inhibiting effect of sera on the activity of thyroid 5'-deiodinase was higher in patients with anti-thyroid peroxidase antibodies compared to antibody negative cases (P<0.01). An inverse correlation was shown between the levels of anti-thyroid peroxidase antibodies and the decreased activity of thyroid 5'-deiodinase (r=-0.6111, P<0.02) in patients with low FT₃.

Conclusion
The low FT₃ or FT₃/FT₄ ratio observed supports the hypothesis that euthyroid sick syndrome is often present in SSc. Anti-thyroid antibody is also frequently detected in SSc and the positive sera inhibit the activity of thyroid 5'-deiodinase, which can contribute to the low FT₃ or FT₃/FT₄ ratio. Anti-thyroid peroxidase antibodies may play an additive role in the development of low FT₃ levels via the inhibiting effect of thyroid 5'-deiodinase. The low FT₃ levels may directly influence the already impaired microcirculation in SSc by increasing the systemic vascular resistance.

Key words
Euthyreoid sick syndrome, systemic sclerosis, deidodinase, anti-thyroid antibody.
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Introduction
Systemic sclerosis (SSc) is a connective tissue disease characterized by fibrosis, obliteratorve vasculopathy and microvascular lesions. The presence of abnormal thyroid function and anti-thyroid antibodies has been demonstrated in SSc (1, 2). The most frequent thyroid abnormality in SSc is hypothyroidism (3-6). Low T3 syndrome has not been described as a thyroid function abnormality in patients with SSc, however (7). Our previous study revealed a strong correlation between the levels of anti-thyroid peroxidase (TPO) antibodies and the low T3 in SSc (8, 9). These findings suggested that the association of autoimmune thyroiditis with SSc could be responsible for the low levels of T3.

Recent data on thyroid hormone metabolism highlight the importance of the deiodinases enzymes (10). The role of deiodinase enzymes in the thyroid hormone metabolism is well-known, as well as their effects in euthyroid sick or low T3 metabolism is well-known, as well as their effects in euthyroid sick or low T3 syndrome (11). Three deiodinase isotypes are involved in the maintenance of the intracellular T3 concentration (DI, DII, DIII). The conversion of T4 to active hormone T3 is mediated by 5'-deiodinases (DI and DII) as 5' outer ring-deiodination (12). The conversion of T4 to inactive hormone rT3 is mediated by 5'-deiodinases (DI and DIII) as 5 inner ring-deiodination. The decreased levels of T3 in SSc can arise from a defect of T3 deiodination via different effects (cytokines, lipid peroxidation, drugs) on the enzymatic activities of the deiodinases (13,14).

The aim of our present study was to demonstrate the relationship between the presence of anti-TPO antibodies and the low levels of FT3 hormone through the inhibiting effect of sera on the activity of thyroid 5'-deiodinase in patients with SSc. Patients with undifferentiated connective tissue disease (UCTD), and secondary and primary Raynaud’s phenomenon (RP) seemed to be reasonable groups to use for comparison (15, 16). RP is often the first clinical sign of SSc and it may preceede the onset of scleroderma by several years (17).

Materials and methods

Subjects
Patients with SSc (n=21), UCTD (n = 12), or secondary (n = 19) or primary (n =11) RP and 16 control patients were investigated. The signs and symptoms in the SSc patients (mean age 45 ± 12 yrs, one male) were screened according to a standard protocol (18). Three patients with diffuse (dcSSc) and 18 patients with limited cutaneous (lcSSc) SSc were investigated. Eight patients (2 with dcSSc and 6 with lcSSc) had anti-topoisomerase I (anti-Scl-70) antibody. Only 3 patients (all with lcSSc) exhibited anti-centromere antibody (ACA).

The diagnosis of the patients with undifferentiated connective tissue disease (UCTD) (mean age 39±17 yrs, one male) was based on the criteria used by previous investigators including Calvo-Alén et al. (19) and Alarcón et al. (20) with substantial modifications. UCTD comprises a group of patients with a systemic disorder that lacks definitive characteristics of any specific, well-defined connective tissue disease. Well defined connective tissue diseases [predominantly systemic sclerosis (SSc), systemic lupus erythematosus (SLE), rheumatoid arthritis or Sjögren’s syndrome] may develop later in many cases. The majority of patients with UCTD tend to remain in a permanently stable clinical-laboratory condition during follow up. Cases with UCTD should exhibit one of the following principle symptoms: (1) Raynaud’s phenomenon (RP); (2) unexplained non erosive, symmetric polyarthritis; and (3) isolated keratoconjunctivitis sicca, accompanied by at least 3 of the following signs or symptoms: (1) Raynaud’s phenomenon; (2) arthritis or arthralgia; (3) rash/dermatitis, alopecia, oral ulcers, or UV light sensitivity; (4) keratoconjunctivitis sicca; (5) lung fibrosis, alveolitis and/or decreased diffusing capacity and decreased vital capacity; (6) dysphagia/pyrosis with oesophageal dysmotility; (7) cardiac involvement; (8) pleuritis/pericarditis; (9) otherwise unexplained peripheral neuropathy/central nervous system symptoms; (10) proteinuria, cylinduria, hematuria or signs of tubular nephropathy; (11) sclerodactyly, swollen fingers, oedema of the fingers or skin ulcers; (12) otherwise unexplained elevated ESR/CRP; (13) positive antinuclear antibody test with homogeneous, fine speckled, nucleolar, anticytobody staining pattern (on HEp-2 cells by indirect immunofluorescence) or specific
autoantibody positivity (anti-dsDNA, anti-SS-A, anti-Rnp, anti-centromere, anti-topoisomerase I, repeatedly positive anti-cardiolipin IgG or IgM by ELISA test); and (14) abnormal nailfold capillaroscopy findings compatible with the presence of scleroderma capillary pattern.

Secondary RP (mean age 42±12 yrs, 17 females, 2 males) was based on the criteria of LeRoy and Medsger (21). In our present study, a special group of patients with RP was formed. Clinically all of these cases exhibited RP as the exclusive predominating clinical symptom but they had no signs of internal organ involvement. In addition to the clinical investigation, chest radiograph, spirometry/diffusion capacity, Schirmer’s test, barium swallow or gastrofiberoscopy, urine analysis, and serum creatinine kinase were performed in all cases. Thus, these cases in addition to Raynaud phenomenon also had either antinuclear antibody positivity, a sclerodermic capillary pattern or digital pitting, ulcerations or gangrene, but they definitely did not exhibit any internal organ manifestations (such as pulmonary interstitial changes, oesophageal dysmotility/reflux oesophagitis, renal symptoms). The expression “patients with secondary RP” is used for this special group of RP patients.

The patients with primary RP (mean age 37 ± 13 yrs, no males) had exclusively RP and were used as negative controls. None of these patients received corticosteroid therapy at the time of the study. Euthyroid goitre in the patient’s medical history was found in 4 cases of SSC, 5 cases of UCTD, 2 cases of secondary RP and 3 cases of primary RP.

Sixteen patients with acute cardiovascular disease (7 to 10 days after myocardial infarction) (mean age 30 ± 10 yrs, 3 males) were investigated as controls. The levels of thyroid hormones in the control group reflected the changes like an euthyroid sick syndrome.

Methods

Preparation of thyroid fractions

Human thyroid tissue was obtained during an operation from a patient with goitre. The homogenate was centrifugated at 800 g and different fractions were made by various centrifugation (10,000 g, 100,000 g). The activity of 5'-deiodinase was detected in the thyroid supernatant of 100,000 g. Human thyroglobulin (Htg) fraction was purified from post-microsomal saline extracts (supernatant of 70,000 g) of toxic goitres by ammonium sulphate fractionation and sepharose 6B (Pharmacia, Sweden) chromatography after Hamada et al. (22). Thyroid peroxidase (TPO) fraction was prepared from porcine thyroid tissue after Taurug et al. using digestion with trypsin (35 kU/g protein, Merck, Germany), ion-exchange chromatography (DE-52, Whatman, UK) and solubilization with deoxycholate in the 100,000 g supernatant (23). The protein concentration was measured by Lowry’s method and stored (without protease inhibitor) frozen at -40°C (24).

Outer ring iodothyronine deiodinase assay

Thyroid homogenates (11 µg protein/50 µl) were incubated with 20 µl undiluted and non-decomplemented patient sera and 50 µl dithiothreitol (20 mM, DTT, Reanal, Hungary) and 50 µl 125I-T₄ (100,000 cpm, Isotope Institute, Budapest, Hungary) for 90 min at 37°C in a final volume of 250 µl (25). The reaction was stopped by 100 µl bovine serum albumin (5% BSA, Sigma, USA), and immediately followed by the addition of 300 µl trichloroacetic acid (10%, Reanal, Hungary). The supernatant containing acid-soluble radiiodine was separated from the precipitate containing iodothyronines and both were measured in a gamma counter (Gamma NZ 322, Hungary). The results were extrapolated at 1 pmol/l T₄ in the patient serum. To determine the Km of iodothyronine deiodinase for T₄ 0, 0.6, 1.8, 3.8 ng/100 ml non-radioactive T₄ were added to the thyroid supernatant that already contained endogenous T₄ (1.7 nmol/l). The activity of thyroid 5'-deiodinase was expressed as pmol of T₄ converted per mg/min protein. The maximum velocity (V_max) and Km (nM) were calculated using Line-weaver-Burk analysis.

Indirect enzyme linked immunosorbent assay (ELISA)

96-well plates (Dynatech Immulon™, USA) were coated with 0.5 µg/100 µl antigen fractions (diluted in carbonate buffer, 0.2 M, pH 9.6) and incubated overnight at 4°C (26). Thyroglobulin and thyroid peroxidase antigen fractions were used for the detection of anti-Htg and anti-TPO antibodies, respectively. 100 µl patient sera (diluted 1:100) were added to the wells of the plate for 2 hr at room temperature. Goat anti-human IgG antibody conjugated with horseradish peroxidase (SIGMA, USA) as secondary antibody (indilution 1: 5000) was chosen for the detection of the primary autoantibodies. O-phenylene-diamine (OPD, Reanal, Hungary) was used as substrate and the optical density (O.D.) values were read at 492 nm (MPR4 MicroPlate automatic ELISA reader, Belgium). The results were given as an ELISA index: the ratio of O.D. of the patient sample and the mean O.D. of controls. When the O.D. value of the given sample exceeded the control value of mean±2SD, the result was regarded as positive for the investigated antibody. ELISA indices (mean ± SD) in the control group were as follows: for anti-Htg 0.89 ± 0.21 and for anti-TPO 0.94 ± 0.2.

Determination of thyroid hormones

The levels of thyroid hormones (FT₄, FT₃, TSH) were determined with a luminoscence immunoassay (LIA-MAT, Byk Sangtec, Germany). The normal values for thyroid hormones were as follows: for FT₄ 7.72 - 23.18 pmol/l, for FT₃ 2.5 - 4.5 pg/ml, and for TSH 0.3 - 3 mIU/l.

Statistics

Student’s paired t-test and linear correlation with GraphPad Prism (USA) were used for the statistical analysis.

Results

Two patients (from subset of dcSSc and UCTD) had subclinical hypothyroidism and one patient with SSC had subclinical hyperthyroidism at the time of the investigation. Low levels of FT₃ (FT₃ < 2.5 pg/ml) were detected in 9 cases of SSC, one case of UCTD, 10 cases of secondary and 8 cases of primary RP (Table 1). No difference was demonstrated between the subsets of dcSSc and lcSSc either in the levels of FT₃ (2.95 ± 0.79 pg/ml vs 2.99 ± 1.59 pg/ml, NS) or in the ratio of FT₃/FT₄ (0.16±0.02 vs 0.21 ±0.14, NS). The decrease in the levels of FT₄ was significant in primary RP compared to the controls (2.21 ± 0.84 vs 3.44±0.42 pg/ml, P < 0.002). The levels
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Table I. The levels of thyroid hormones in patients with systemic sclerosis (SSc), undifferentiated connective tissue disease (UCTD), secondary and primary Raynaud’s phenomenon (RP), and controls.

<table>
<thead>
<tr>
<th>Patient groups</th>
<th>Age (yrs.) (mean ± SD)</th>
<th>TSH (0.3 - 3 mIU/l)</th>
<th>FT4 7.72 - 23.18</th>
<th>FT3 2.5 - 4.5</th>
<th>FT3/FT4</th>
<th>Anti-thyroglobulin Ab ELISA index mean ± SD (pos. cases / total)</th>
<th>Anti-thyroid peroxidase Ab ELISA index mean ± SD (pos. cases / total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSc (n = 21)</td>
<td>45 ± 12</td>
<td>1.29 ± 1.21</td>
<td>16.14 ± 3.52a</td>
<td>2.99 ± 1.5</td>
<td>0.2 ± 0.13c</td>
<td>1.35 ± 0.44d (12/21)</td>
<td>2.06 ± 0.81a (17/21)</td>
</tr>
<tr>
<td>UCTD (n = 12)</td>
<td>39 ± 17</td>
<td>1.2 ± 1.86</td>
<td>16.42 ± 2.33d</td>
<td>4.59 ± 3.01</td>
<td>0.29 ± 0.21</td>
<td>1.13 ± 0.36 (3/12)</td>
<td>1.43 ± 0.46e (7/12)</td>
</tr>
<tr>
<td>Secondary RP</td>
<td>(n = 19)</td>
<td>42 ± 12</td>
<td>1.19 ± 0.49</td>
<td>15.08 ± 2.96</td>
<td>3.63 ± 5.03</td>
<td>0.23 ± 0.28</td>
<td>1.15 ± 0.5 (5/19)</td>
</tr>
<tr>
<td>Primary RP</td>
<td>(n = 11)</td>
<td>37 ± 13</td>
<td>3.33 ± 6.25</td>
<td>10.15 ± 2.44</td>
<td>2.21 ± 0.84b</td>
<td>0.23 ± 0.11d</td>
<td>0.8 ± 0.33 (1/11)</td>
</tr>
<tr>
<td>Controls</td>
<td>(n = 16)</td>
<td>30 ± 10</td>
<td>1.88 ± 0.78</td>
<td>9.28 ± 1.7</td>
<td>3.44 ± 0.42</td>
<td>0.38 ± 0.08</td>
<td>0.89 ± 0.21</td>
</tr>
</tbody>
</table>

*p < 0.0001; p < 0.002; p < 0.001; p < 0.01; p < 0.004 in comparison with controls using Student’s paired t-test.

The addition of patient sera to the thyroid fraction decreased the activity of thyroid 5'-deiodinase significantly in the SSc (2.88 ± 0.61 pmol/mg/min, P < 0.0001), UCTD (2.86 ± 0.38 pmol/ml/min, P < 0.0004) and secondary RP (3.23 ± 0.81 pmol/ml/min, P < 0.0001) in comparison with the control (4.99 ± 1.04 pmol/ml/min) (Fig. 1). The V_max and K_m of the activity of 5'-deiodinase in the thyroid fraction was determined using Lineweaver-Burk analysis (V_max = 1.39 pmol/ml/min, K_m = 6.82*10^-3 M).

The anti-TPO positive (N = 33) sera inhibited the activity of thyroid 5'-deiodinase rather than sera of anti-TPO negative (N = 24) (2.96±0.52 vs 3.61±1.15 pmol/ml/min, P < 0.01) (Fig. 2). An inverse correlation was demonstrated between the levels of anti-TPO antibodies and the activity of thyroid 5'-deiodinase in the patients with decreased FT3 (>4.5 pg/ml) in comparison with controls using Student’s paired t-test.

Fig. 1. Effect of sera from patients with systemic sclerosis (SSc), undifferentiated connective tissue disease (UCTD), or Raynaud’s phenomenon (RP) (either the secondary or primary form) and controls on the activity of thyroid 5'-deiodinase. The mean ± SD was the follows: for controls (n = 16) 4.99 ± 1.04 pmol/mg/min; for SSc (n = 21) 2.88 ± 0.61 pmol/mg/min; for UCTD (n = 12) 2.86 ± 0.38 pmol/mg/min; for secondary RP (n = 19) 3.23 ± 0.81 pmol/mg/min and for primary RP (n = 11) 4.65 ± 0.84 pmol/mg/min.
Discussion

Anti-thyroid antibodies, as a sign of a usually subclinical thyroiditis, have been described in SSc (1, 6, 8, 27). The high levels of anti-TPO and anti-Htg with decreased levels of FT3 and a low FT3 / FT4 ratio were also found in SSc as indicated by our previous data (9). The hallmark of the euthyreoid sick syndrome, the decreased ratio of FT3 / FT4 (< 0.2) is a sensitive indicator of deiodinase activity. The low FT3 / FT4 ratio can be regarded as a consequence of the inhibition of 5'-deiodinases (DI and DII). 20% of the circulating T3 is secreted by thyroid, which has DI and DII deiodinase isotypes. Both are characterised as selenoenzymes (3, 28, 29). Low FT3 levels represent a hypothyroid-like effect on the cell functions and are predicted by the activities of thyroid and non-thyroid tissue specific 5'-deiodinases. 5-deiodinase (DIII) catalyzes T4 to rT3 conversion which can be associated with elevated FT3.

Our recent findings demonstrated that the sera of patients with SSc, UCTD and secondary RP can inhibit the activity of thyroid 5'-deiodinase. The tissue-specific activities of deiodinases (thyroid, liver, kidney, skin, cardiac and skeletal muscles, brain) could be decreased by cytokines (IL-6, TNFα), drugs (glucocorticoids, propranolol, iodinated radiocontrasts) and nutrition deficiency (selenium) (13, 30). Elevated levels of IL-6 and TNFα decreased the activity of thyroid 5'-deiodinase in our patients with SSc (31). Selenium deficiency, which is an additional factor to the blocking effects on deiodinase enzymes, has also been described in SSc (32). Patients with low FT3 exhibit the clinical signs of high systemic vascular resistance, low cardiac output and impaired oxygen uptake and metabolism of skeletal muscle (33). It seems that the low level of FT3 with its direct vascular effect may contribute to the impaired tissue perfusion not only in cardiovascular diseases but also in SSc and primary RP (34, 35). Ischemic-reperfusion injury is defined as one of the important mechanisms in the pathogenesis of SSc (36). The development of low FT3 without decreased activity of thyroid 5'-deiodinase in primary RP seems to be a consequence of the activities of non-thyroid tissue specific 5'-deiodinases.

The strong correlation between anti-TPO positivity and the inhibiting effect of patient sera on the activity of thyroid 5'-deiodinase suggest that autoimmune phenomena are involved in the low levels of FT3 via autoantibodies against the proteins of 5'-deiodinases. TPO is the one of the major thyroid antigens and plays a key role in the iodination of tyrosyl residues in thyroglobulin so that in the thyroid hormone production. Anti-TPO antibodies are able to inhibit the enzyme activity of TPO (37). Autoantibodies against the proteins of thyroid 5'-deiodinases (DI and DII) may be present in autoimmune diseases such as SSc and UCTD. On the other hand, the idea that antibodies may be directed against the same epitopes of TPO and 5'-deiodinase (DII) seems to be speculative. However, 9 anti-TPO positive cases with elevated FT3 out of the all studied patient groups were found to have decreased 5'-deiodinase activity. These cases suggested the possibility of autoantibodies directing to the enzyme protein with the both...
activities of 5'- and 5-deiodinases in the thyroid tissue (DI).

The high prevalence of low FT3 with the low ratio of FT3 / FT4 in primary RP without any association with anti-TPO antibodies suggest the role of non-immune phenomena in the induction of decreased FT3. There are some recent data that the effect of low FT3 on the vascular lesions of RP has been improved by the treatment of triiodothyronine (38). Our data indicate that the presence of certain thyroid-related autoantibodies in SSc and UCTD may influence the activity of thyroid 5'-deiodinase. The decrease in the levels of FT3 in the positive cases for anti-TPO correlated with the inhibitory effect of these sera on the activity of thyroid 5'-deiodinase. Euthyroid sick syndrome is present in SSc and in primary RP. Inhibition of the activities of non-thyroid tissue specific 5'-deiodinase may also be play a role in the low levels of FT3, as was found in patients with primary RP. The decreased levels of FT3 is an important factor for the development of vascular lesions. The low FT3 represents a hypothyroid-like effect. The direct influence of low FT3 on the vascular system contributes to the impaired tissue microcirculation that already is present in SSc.

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