CLINICAL STUDY

Increased serum CXCL10 in Graves' disease or autoimmune thyroiditis is not associated with hyper- or hypothyroidism *per se*, but is specifically sustained by the autoimmune, inflammatory process

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Abstract

Objective: Serum CXCL10 (an interferon- γ -inducible chemokine) levels (sCXCL10) are increased in several autoimmune conditions, including Graves' disease (GD) and autoimmune thyroiditis (AT). Longitudinal assessment of sCXCL10 in autoimmune hypo- or hyperthyroidism has not yet been performed.

Design and methods: We longitudinally assayed sCXCL10 in the following groups:

1. thirty-three GD and 11 toxic nodular goiter (TNG) patients when hyperthyroid (Hyper) and when reaching euthyroidism (Eu) with methimazole therapy (MMI)
2. sixty-six AT (33 hypothyroid (Hypo) and 33 Eu) patients, basally and after reaching EU (for Hypo) with levothyroxine (L-T₄) therapy
3. twenty-two patients with thyroid cancer (CA) under L-T₄-suppressive treatment, of whom 11 were re-evaluated after L-T₄ withdrawal for diagnostic WBS, and 11 after recombinant TSH (rhTSH) administration
4. thirty-three healthy controls.

Results: At initial evaluation, Hyper GD and AT (Hypo significantly higher than Eu) showed significantly higher mean sCXCL10 than all other groups. MMI treatment led to a significant decrease in sCXCL10 only in GD (not in TNG), while restoration of Eu, in Hypo AT, by L-T₄ was not accompanied by significant sCXCL10 change. CA showed sCXCL10 comparable to controls, and both Hypo after L-T₄ withdrawal and rhTSH injection had no effect on sCXCL10.

Conclusions: Treatment of Hyper leads to a significant decrease in sCXCL10 only in GD, and this probably depends upon the MMI immunomodulatory effect. L-T₄ correction of Hypo is not accompanied by significant modification of sCXCL10 in AT. Increased sCXCL10 is not associated with Hyper or Hypo *per se*, but is specifically sustained by the autoimmune inflammatory event occurring in both GD and AT.

European Journal of Endocrinology 154 651–658

Introduction

Chemokines are a group of peptides of low molecular weight that induce the chemotaxis of different leukocyte subtypes (1). The major function of chemokines is the recruitment of leukocytes to inflammation sites, but they also play a role in tumoral growth, angiogenesis and organ sclerosis (2, 3). At present, more than 50 chemokines have been described, classified into four major families (1). The CXC chemokines inducible

by interferon (IFN)- γ (CXCL9, CXCL10 and CXCL11) are associated with Th1-mediated immune responses; among them, CXCL10 is a prototype, and its serum levels are increased in several endocrine autoimmune conditions (4–9). Recent experimental evidence has demonstrated that CXC chemokines, particularly CXCL10, play an important physiopathologic role in the initial phases of autoimmune thyroid disorders (AITD) (7, 10, 11). Briefly, in patients with Graves' disease (GD), the CXCR3 receptor was found to be highly

expressed in endothelial cells as well as in infiltrating inflammatory cells, while CXCL10 was detected not only on these cells but also on thyrocytes (7, 12). Human thyrocytes in primary culture produce large amounts of CXCL10 when stimulated by IFN- γ (10), and increased expression of CXCL10 and CXCL9 was also observed in thyroid tissue specimens obtained from subjects affected by autoimmune thyroiditis (AT) by immunohistochemistry (10). In summary, previous studies have demonstrated a role for CXC chemokines in GD by evaluation of intraglandular mRNA or protein expression levels (11, 13). The following step for the evaluation of the role played by chemokines in AITD has been brought about by retrospective transversal studies evaluating serum CXCL10 in large series of GD and AT patients (7–9). Serum CXCL10 has been found to be increased in both GD and AT in comparison to healthy controls, and subsequent evaluation of circulating chemokines in relation to the clinical phenotype demonstrated a significant inverse correlation between circulating CXCL10 levels and disease duration in GD (7), together with a strong association with hypothyroidism and thyroid hypoechogenicity in AT (8, 9), while no significant relationship was observed for serum thyroid autoantibodies in both conditions. To our knowledge, no longitudinal study has evaluated systematically the IFN- γ -inducible chemokine status in patients with various types of thyroid disorders in relation to the presence of hypo- or hyperthyroidism and subsequent treatments. Given that comparisons between data obtained by tissutal quantification of chemokine expression and assessment of circulating levels must always be careful, and that, once proven useful, serum CXCL10 measurement could represent an easily assayable marker for clinical management of AITD patients, longitudinal clinical trials seem to be

required in order to assess the potential benefit of CXCL10 assay in the clinical management of such patients.

Therefore, the aim of the present study was to evaluate longitudinally serum CXCL10 levels (sCXCL10) in patients with AT, GD, toxic nodular goiter (TNG) and thyroid cancer (CA), in hypo- or hyperthyroidism, and after restoration of euthyroidism, to relate serum CXCL10 to thyroid function and the type of treatment.

Subjects and methods

Patients

From the outpatient clinic, we prospectively studied the following groups:

1. Thirty-three consecutive Caucasian patients with hyperthyroid GD, without clinical signs or symptoms of GD ophthalmopathy (Table 1). The patients were referred to us by general practitioners or other hospitals because of the presence of hyperthyroidism or of circulating thyroid autoantibodies, or clinical suspicion of thyroid disorder. The diagnosis of GD (14) was established from the clinical presentation (presence of a diffuse goiter, varying in size from normal to very large), thyroid hormones and thyroid autoantibodies measurements (presence of antithyrotropin-receptor autoantibodies (TRAb) and/or thyroid ultrasonography (decreased, dyshomogeneous echogenicity, and diffuse goiter). Most of these patients had goiter (57%); the others showed normal thyroid volume. TRAb were present in 85% of patients. A minority of patients (6%) underwent fine-needle aspiration (FNA) of thyroid nodules to exclude

Table 1 Thyroid status of control subjects and patients with Graves' disease, autoimmune thyroiditis, toxic nodular goiter or thyroid cancer.

	Controls	Graves' disease	Thyroiditis	Toxic nodular goiter	Thyroid cancer	P
n	33	33	66	11	22	
Age (years)	42 $\pm$ 15	44 $\pm$ 16	43 $\pm$ 17	46 $\pm$ 11	44 $\pm$ 15	ns
Gender (M/F)%	20	20	20	18	18	ns
Thyroid volume (ml)	10 $\pm$ 3	31 $\pm$ 26°	12 $\pm$ 11	29 $\pm$ 22°	—	0.0001
Hypoechoic (%)	0	64	60	0	—	0.0001
Hypervascular (%)	0	73	33	0	—	0.0001
Serum TSH (μ U/ml)	1.6 $\pm$ 0.7	0.04 $\pm$ 0.05°	3.4 $\pm$ 6.4#	0.07 $\pm$ 0.07°	0.03 $\pm$ 0.06°	0.006
FT ₄ (ng/dl)	9.3 $\pm$ 1.6	16.3 $\pm$ 11.4°	8.4 $\pm$ 2.5	15.6 $\pm$ 10.7°	11.2 $\pm$ 2.1°	0.0001
FT ₃ (pg/ml)	3.9 $\pm$ 0.9	6.7 $\pm$ 3.1°	3.4 $\pm$ 1.1	5.9 $\pm$ 2.8°	3.6 $\pm$ 0.7	0.0001
AbTPO (UI/ml)	9 $\pm$ 11	211 $\pm$ 332*	473 $\pm$ 328*	10 $\pm$ 7	3 $\pm$ 7	0.0001
AbTg (UI/ml)	10 $\pm$ 9	189 $\pm$ 552*	349 $\pm$ 287*	11 $\pm$ 10	9 $\pm$ 11	0.0007
TRAb (UI/ml)	0	21 $\pm$ 38§	0	0	—	0.0001
AbTPO positivity (%)	0	45	80	0	0	0.0001
AbTg positivity (%)	0	36	77	0	0	0.0001
CXCL10 (pg/ml)	75 $\pm$ 34	161 $\pm$ 106*	149 $\pm$ 89*	96 $\pm$ 26	63 $\pm$ 43	0.0001

°P < 0.05 or less vs controls or vs autoimmune thyroiditis.

*P < 0.05 or less vs controls or vs thyroid cancer or vs toxic nodular goiter.

#P < 0.05 or less vs controls.

§P < 0.05 or less vs the others.

- the presence of CA or lymphoma; in these cases, cytology excluded the presence of malignancy. All were untreated hyperthyroid patients (thyroid-stimulating hormone (TSH) of $<0.1 \mu\text{U/ml}$, associated with high levels of free tri-iodothyronine (FT_3) and/or free thyroxine (FT_4). All GD patients were given methimazole (MMI) therapy (and, when necessary, beta-blockers to control cardiac rate), and thyroid function was re-evaluated after 1, 2 and 3 months to obtain a condition of euthyroidism (TSH, FT_3 and FT_4 in the normal range) (31 were in euthyroidism with MMI after 3 months of therapy).
2. Sixty-three patients with chronic AT (Table 1). Among them, 33 patients were hypothyroid and 33 were euthyroid, and were matched by gender and age (± 5 years) with GD patients. The diagnosis of AT (14) was established from the clinical presentation (presence of a firm goiter, varying in size from small to very large, with a lobulated surface), thyroid hormones and thyroid autoantibodies measurements, and/or thyroid ultrasonography (decreased, dyshomogeneous echogenicity). Hypothyroid patients (TSH $>3.5 \mu\text{U/ml}$) were given levothyroxine (L-T_4) substitutive therapy to correct hypothyroidism. Patients were re-evaluated after 6 weeks, and, if necessary, the L-T_4 treatment was adjusted again to obtain the condition of euthyroidism (TSH $>0.3 \mu\text{U/ml}$ and $<3.5 \mu\text{U/ml}$, with FT_3 , and FT_4 in the normal range). Serum CXCL10 was re-evaluated in both euthyroid AT (without any kind of treatment) and hypothyroid AT (all were in euthyroidism with L-T_4), after 3 months from the initial evaluation.
  3. Eleven patients affected by toxic nodular goiter (TNG), consecutively recruited in the same period, matched by gender and age (± 5 years) with 11 of the previously described GD patients (Table 1). All patients were hyperthyroid for the presence of autonomous nodules (by ultrasound and thyroid scan), and most of them had a thyroid volume under 20 ml (64%). All these patients underwent FNA to exclude CA; cytology confirmed the absence of malignancy. All TNG patients were given MMI (and, when necessary, beta-blockers to control cardiac rate), and thyroid function was re-evaluated after 1, 2 and 3 months to obtain the condition of euthyroidism (all were in euthyroidism with MMI after 3 months of therapy).
  4. Eleven patients with CA, matched by gender and age (± 5 years) with 11 GD patients, who had undergone total thyroidectomy, and ablation of the residual tissue with ^{131}I , and in whom whole-body scan (WBS), neck ultrasonography and thyroglobulin indicated absence of residual disease, in treatment with L-T_4 at suppressive dosage of TSH (TSH $<0.1 \mu\text{U/ml}$, with FT_3 , and FT_4 in the normal range). Serum CXCL10 levels were re-evaluated in the same patients 1 month after L-T_4 withdrawal, in hypothyroidism, before diagnostic WBS.
  5. Eleven patients with CA, matched by gender and age (± 5 years) with 11 GD patients, who had undergone total thyroidectomy, and ablation of the residual tissue with ^{131}I , and in whom WBS, neck ultrasonography and thyroglobulin indicated absence of residual disease, in treatment with L-T_4 at suppressive dosage of TSH. Serum CXCL10 levels were evaluated in these patients before injection of recombinant human TSH (rhTSH) (Thyrogen; Genzyme Transgenics, Cambridge, MA, USA). Two doses of 0.9 mg thyrogen, administered i.m., were given once daily for the first 2 days. Twenty-four hours later, at 48 h of study, 5 mCi ^{123}I were administered orally, and WBS was performed. Serum CXCL10 was evaluated 24 h (1 day) after the injection of the first vial of rhTSH and 24 h after the injection of the second vial of rhTSH (2 days/48 h after the first one).
  6. A further control group (controls, $n = 33$) consisted of a random sample of the general population, matched by gender and age (± 5 years) with the 33 GD patients, from the same geographic area (15), for whom a complete thyroid workup (history; physical examination; TSH, FT_3 , FT_4 , AbTg and AbTPO antibodies measurements; and ultrasonography) was available that excluded the presence of thyroid disorder.

All study subjects gave their informed consent to the study, which was approved by the local ethics committee.

Ultrasonography of the neck and FNA

Neck ultrasonography was performed with a probe (Esaote, AU5, with a sectorial 7.5 MHz transducer) in GD, AT and TNG patients and controls by the same operator, who was unaware of the results of thyroid hormones, autoantibodies and CXCL10 measurements. Thyroid volume was calculated by the ellipsoid formula, as previously described (14, 16, 17). The presence of hypoechoic and dyshomogeneous echogenicity was arbitrarily rated at three levels (0 = normal echogenicity; 1 = slight hypoechoic and dyshomogeneous; 2 = severely hypoechoic and dyshomogeneous) to evaluate structural abnormalities of thyroid tissue associated with thyroid autoimmunity (18, 19). The presence of thyroid nodules was recorded, and nodules with a diameter over 10 mm were submitted to ultrasonography-guided FNA, which was performed by the same operator, using the free-hand method, as previously described (17, 19).

Thyroid blood flow

Thyroid blood flow (TBF) by color-flow Doppler (CFD) was studied in all patients (20). The CFD pattern was classified as follows:

- normal (or type 0): TBF limited to peripheral thyroid arteries
- type I: TBF mildly increased
- type II: TBF clearly increased
- type III: TBF markedly increased (20).

Laboratory evaluation

Thyroid function and thyroid autoantibodies were measured as previously described (14). Circulating FT₃ and FT₄ were measured by commercial RIA kits (Amersham-MAB FT₃/FT₄ Kit; Amersham). Serum TSH (DiaSorin, Stillwater, MN, USA), AbTPO and AbTg (ICN Pharmaceuticals, Costa Mesa, CA, USA) were evaluated by IRMA methods. TRAb autoantibodies were measured in patients (GD, AT and TNG) and controls by radioreceptor assay (Radim, Pomezia, RM, Italy) (normal range 0–1 UI/ml). For AbTg and AbTPO, positivity was set at > 50 and > 10 UI/ml respectively.

CXCL10 ELISA

Serum CXCL10 levels were assayed by a quantitative sandwich immunoassay with a commercially available kit (R&D Systems, Minneapolis, MN, USA), with a sensitivity of 0.41–4.46 pg/ml and a mean minimum detectable dose of 1.67 pg/ml. The intra- and interassay coefficients of variation were 3.0% and 6.9% respectively.

Data analysis

Values are given as mean $\pm$ s.d. for normally distributed variables; otherwise, as median and interquartile range. Mean group values were compared by one-way analysis of variance (ANOVA) for normally distributed variables; otherwise, by the Mann–Whitney *U* or Kruskal–Wallis test. Proportions were compared by the chi-square test. Post-hoc comparisons on normally distributed variables were carried out with the Bonferroni–Dunn test.

Results

The demographic and clinical features of patients and controls are reported in Table 1. Mean sCXCL10 were significantly higher in GD and AT than in the other group of patients.

The mean CXCL10 level was significantly higher in patients with hypothyroid than in euthyroid patients with AT, as previously reported (8, 9) (Fig. 1). A slight, but not significant decrease of serum CXCL10

Figure 1 Mean serum CXCL10 levels in hypothyroid patients with autoimmune thyroiditis (AT) were significantly ($P < 0.05$, by ANOVA) higher than in euthyroid subjects.

levels after 3 months was observed both in euthyroid patients with AT (off therapy) (Fig. 2a) and in hypothyroid AT patients after reaching euthyroidism with L-T₄ treatment (Fig. 2b).

In patients with CA, sCXCL10 was not significantly different from controls, and hypothyroidism did not induce any significant change (Fig. 3). Similarly, no significant change of serum CXCL10 levels was observed after rhTSH (Fig. 4).

GD patients with untreated hyperthyroidism had significantly higher CXCL10 levels than the same patients treated for 3 months with MMI in euthyroidism

Figure 2 No significant change of serum CXCL10 levels was observed in either euthyroid patients with AT (a) (off therapy) or hypothyroid AT patients after reaching euthyroidism with L-T₄ treatment (b), after a period of 3 months.

Figure 3 A significant increase of (a) serum TSH ($P < 0.01$, by ANOVA), and comparable (b) serum CXCL10 before (euthyroid) and after (hypothyroid) withdrawal of L-T₄ therapy for 1 month in thyroidectomized patients with thyroid papillary cancer.

(Fig. 5a). Hyperthyroid TNG patients had slightly but not significantly higher sCXCL10 than the same patients treated with MMI for 3 months in euthyroidism (Fig. 5b).

Figure 4 Serum CXCL10 levels, slightly but not significantly, increase in patients 1 day (24 h after injection of the first vial of rhTSH) and 2 days (24 h after injection of the second vial of rhTSH, and 48 h after the first one) after recombinant TSH (rhTSH) injection with respect to basal (0); a significant elevation is observed for serum TSH (a).

Figure 5 A significant decrease of serum CXCL10 levels ($P < 0.05$ by ANOVA) was observed in Graves' disease patients after 3-month treatment with methimazole (MMI) when reaching euthyroidism with respect to the basal level (before) (a), while a slight, but not significant, decrease of serum CXCL10 levels was observed in toxic nodular goiter patients (TNG) after 3-month treatment with MMI when reaching euthyroidism with respect to the basal level (before) (b).

Discussion

The results of the present study confirm that CXCL10 serum levels are increased in patients newly diagnosed with GD, and demonstrate a strong association with the hyperthyroid phase of the disease, with a decrease of CXCL10 with restoration of euthyroidism by MMI therapy. The high levels of CXCL10 were not associated with hyperthyroidism per se; in fact, serum CXCL10 levels were significantly lower in TNG than in GD patients showing comparable serum levels of FT₃ and FT₄. The significant decrease of CXCL10 in GD treated with MMI was probably due to an immunomodulatory action of MMI, and not to the correction of hyperthyroidism; in fact, in TNG patients, MMI did not significantly decrease CXCL10 after the achievement of euthyroidism.

Euthyroid patients with AT had significantly higher levels of CXCL10 than controls, and TNG and CA patients. In hypothyroid AT patients, CXCL10 was significantly higher than in euthyroid AT, and did not change with the correction of hypothyroidism; in both groups, serum CXCL10 levels did not change significantly after a 3-month period of observation. Hypothyroidism per se and rhTSH did not significantly change serum CXCL10 in CA patients. These data suggest that high sCXCL10 in AT is related to inflammatory events

connected with AT itself, and that the higher levels observed in hypothyroid AT are a sign of a more aggressive thyroiditis.

The above reported results confirm our previous studies reporting increased serum CXCL10 levels in autoimmune hyperthyroidism and hypothyroidism, demonstrating that the sCXCL10 increase is specifically associated with the autoimmune process and excluding any possible influence of thyroid dysfunction per se. Such observations agree with our previous experience of a statistically significant increase in intrathyroidal CXCL10 mRNA expression in patients undergoing thyroidectomy for GD with respect to toxic goiter and non-toxic goiter thyroidectomized patients (7). However, several serum cytokines have been previously reported to be increased in autoimmune (21–23) as well as non-autoimmune hyperthyroidism (24–26), showing that this change resulted from the chronic effects of thyroid hormone excess, rather than from the accompanying autoimmune, inflammatory condition present in GD. The issue is complex, and conclusions should be drawn cautiously, as several demographic, clinical and therapeutical variables, which have not always been carefully assessed, may generate biased results. For example, very few studies to establish the role of serum cytokines in hyperthyroidism have considered the role of age, which seems to be an important variable significantly and directly correlated with several cytokines, and CXCL10 in particular, as previously demonstrated (27). Therefore, it should be considered that TNG patients are generally older than GD patients. Moreover, serum thyroid hormones, whose levels are generally higher in GD, as well as the use of corticosteroid treatment, which excludes GD patients with ophthalmopathy, should always be taken into account when comparing serum cytokines in GD and TNG patients. Thus, our study group encompassed several kinds of controls, which were all matched for age and serum FT₃ and FT₄; furthermore, the exclusion of patients with GD ophthalmopathy prevented the evaluation of patients receiving corticosteroid therapy. In this view, the comparable sCXCL10 levels observed between sex- and age-matched, healthy controls, hyperthyroid TNG patients and thyroidectomized patients receiving suppressive L-T₄ treatment, strongly suggest that only a minor role, if any, is played by hyperthyroidism per se in determining increased sCXCL10. Therefore, the significant decrease in serum CXCL10 after restoration of euthyroidism in GD patients reported here could be reasonably ascribed to the well-known immunomodulatory effect of antithyroid drugs. In fact, besides its ability to decrease thyroid hormone production (28), MMI appears to interfere with the immunologic abnormalities in GD hyperthyroidism, as shown by the 50% of patients successfully cured by the drug and by the significant decrease of serum antithyroid antibodies observed in most treated patients (28, 29). Therefore, these drugs may have influenced chemokine

levels, especially as the same drugs can reduce cytokine expression by TFC with a final effect of reducing infiltration into the gland (30). Here, however, current data conflict, some authors reporting a reduction of inflammatory cytokines in hyperthyroid GD patients treated with antithyroid drugs (24–26) and others not finding this (21, 22).

Studies with several sex- and age-matched control groups have excluded the role of hypothyroidism per se in determining increased sCXCL10 in AT patients as compared with normal controls, and TNG and CA patients (8, 9). The lack of significant modifications in serum CXCL10 observed in thyroidectomized patients under L-T₄ replacement treatment and 1 month after the withdrawal of the therapy, together with the absence of any effect on CXCL10 induced by rhTSH administration, seems rather conclusive with regard to this point. In this view, the previously reported (8, 9) and here confirmed significantly increased sCXCL10 in hypothyroid AT with respect to euthyroid AT seems of clinical interest, suggesting that raised CXCL10 is not only associated with the autoimmune process itself, but could also be regarded as a marker of more severe thyroiditic process. The role of CXCL10, a clearly Th1-oriented chemokine, in autoimmune process-dependent thyroid destruction, can be assumed in view of its chemoattractant activity for Th1 lymphocytes secreting IFN- γ whose intraglandular production results in thyrocyte apoptosis (31) and severe hypothyroidism (32). Accordingly, the recent observation that high pretransplant sCXCL10 predicts severe acute rejection and allograft failure in patients undergoing renal transplantation suggests that a strongly Th1-enriched environment (CXCL10) may lead to a more severe course and aggressiveness of Th1-mediated autoimmune reactions (33, 34). Thus, it may be reasonable to speculate that euthyroid AT patients displaying the highest sCXCL10 would be those most likely to develop hypothyroidism. However, in our series, no euthyroid AT patient became hypothyroid over the limited period of follow-up (3 months).

In conclusion, our results, obtained in several groups of patients with thyroid dysfunction of different etiology, demonstrate that:

1. treatment of hyperthyroidism leads to a significant decrease in sCXCL10 only in GD (not in TNG), and is likely to depend upon the MMI immunomodulatory effect
2. L-T₄ correction of hypothyroidism is not accompanied by significant modification of sCXCL10 in AT
3. increased sCXCL10 is not associated with hyper- or hypothyroidism per se, but is specifically sustained by the autoimmune, inflammatory event occurring both in GD and AT.

A longer surveillance time is necessary to determine whether euthyroid patients with AT develop hypothyroidism more frequently and/or rapidly in relation to different serum CXCL10 levels.

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Received 14 November 2005

Accepted 9 February 2006