CLINICAL STUDY

Increase of interferon- γ inducible α chemokine CXCL10 but not β chemokine CCL2 serum levels in chronic autoimmune thyroiditis

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Abstract

Objective: To measure serum levels of CXCL10 and CCL2 prototype chemokines of the two major subclass (CXC and CC) in patients with newly diagnosed chronic autoimmune thyroiditis (AT), and relate the findings to the clinical phenotype.

Design and methods: Serum CXCL10 and CCL2 were assayed in 70 consecutive patients with newly diagnosed chronic AT, in sex- and age-matched healthy volunteers ($n = 37$) and in 20 patients with non-toxic multinodular goiter, extracted from a random sample of the general population from the same geographic area.

Results: CXCL10 serum levels were significantly higher in patients with thyroiditis than in controls or multinodular goiter patients, while comparable CCL2 levels were found between groups. CXCL10 levels were significantly increased in hypothyroid patients and in those with an hypoechoic pattern ($P = 0.0004$ and $P = 0.0001$, respectively) while serum CCL2 levels were significantly increased in patients older than 50 years and in those with hypothyroidism ($P = 0.0001$ and $P = 0.03$, respectively). No correlation between CXCL10 and CCL2 serum levels could be demonstrated. CXCL10 and CCL2 were studied separately in relation to clinical features of AT patients. Two separate multiple linear regression models for CXCL10 and CCL2 were performed, including age, thyroid volume, thyroid stimulating hormone (TSH), FT4, anti-thyroid peroxidase (AbTPO), hypoechoic pattern, and the presence of hypervascularity, demonstrating that $\ln$ of serum CXCL10 levels was associated with TSH independently of other possible confounders levels [regression coefficient (R.C.) 0.143 confidence interval (C.I.) (0.042–0.245); $P = 0.0059$], while serum CCL2 were significantly associated only with age [R.C. 5.412 C.I. (3.838–6.986); $P < 0.0001$].

Conclusion: Our results, obtained in a large cohort of newly diagnosed AT patients demonstrate increased CXCL10 especially in hypothyroid patients with a more aggressive disorder, and normal CCL2 serum levels in AT.

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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, which have been classified into four major families (1). So far, only two of these families have been extensively studied and characterized, namely, the CC and CXC chemokines,

according to the organization of positionally conserved cysteine residues. Chemokines of the CC family generally are chemoattractant for T lymphocytes, monocytes, and natural killer (NK) cells while CXC chemokines attract neutrophils and promote their adherence to endothelial cells (4–6). Monocyte chemoattractant protein 1 (MCP-1) is a prototype CC chemokine, playing an important role in innate immunity (7–10). Moreover, CCL2 is also a crucial factor for the development of adaptive T-helper (Th)2 responses by directing the differentiation of Th0 cells to Th2 *in vitro* (11). Among CXC chemokines, CXCL10 similarly

to other non-ELR (glutamic acid-leucine-arginine negative) chemokines, is a poor neutrophil chemoattractant and activator. However, it displays a strong chemoattractant activity for Th1 lymphocytes secreting INF- γ , with several experimental data suggesting that CXCL10 measurement is a reliable marker of aggressive Th1-mediated autoimmune disease (12).

Autoimmune thyroid disease (AITD) with Hashimoto's thyroiditis (HT) and Graves' disease (GD) are the most frequent endocrine autoimmune conditions, both being characterized by diffuse lymphocytic infiltration which is predominant in HT and varies with the type and stage of AITD (13). Cytokine production during autoimmune thyroid disease shows the expression of interleukin (IL)-1, IL-2, IL-6, IL-10, interferon (IFN)- γ and tumour necrosis factor (TNF)- α by infiltrating T cells and macrophages (14–17). In addition, thyroid follicular cells (TFC) themselves produce many cytokines (18–21). More recently chemokines have been identified to play an important role in endocrine autoimmune disease and particular attention has been raised by studies demonstrating both CC and CXC chemokine overexpression in HT and in early phases of GD (22–23).

Subsequent studies have shown that expression of CXCL10 and CCL2 is also stimulated by different proinflammatory cytokines in thyroid follicular cells (TFC) (24, 22) and that both α and β chemokines are overexpressed in GD thyroid tissue (25).

In addition, by immunohistochemistry an increase in CXCL10/IP-10 and CXCL9/Mig was found in thyroid tissue specimens obtained from subjects affected by HT (22).

The possible role of circulating α and β chemokines in AITD has been less extensively studied, with only one previous observation regarding increased serum CCL2 levels in chronic autoimmune thyroiditis (AT) and increased serum CXCL10 in GD patients (23, 26, 27).

Besides their chemotactic activity, chemokines modulate the immune responses through the recruitment of specific T helper subsets and cytokine production. The patterns of cytokines secreted by Th1 and Th2 cells result in particular combinations that specifically drive particular types of immune response (25). Therefore the simultaneous assessment of different chemokines may be potentially of great interest, as demonstrated in multiple sclerosis (MS), a Th1 mediated autoimmune disease, in which CXCL10 and CCL2 display different behaviour in relation to the acute or stable phase of disease (28–30).

To our knowledge, no study has evaluated contemporarily serum α and β chemokines in patients with AT. The aim of the present study therefore was to measure serum levels of CXCL10 and CCL2 prototype chemokines of the two major subclass (CXC and CC) in patients with newly diagnosed chronic AT, and relate the findings to the clinical phenotype.

Patients and methods

Patients

From the outpatient clinic, we selected 70 consecutive Caucasian patients with newly diagnosed chronic AT (Table 1). The patients were referred to us by general practitioners or other hospitals because of the presence of circulating thyroid autoantibodies or hypothyroidism, or clinical suspicion of a thyroid disorder. The diagnosis of AT (31, 32) 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 autoantibody measurements, and/or thyroid ultrasonography (decreased, dyshomogeneous echogenicity). The majority of these patients had a normal thyroid volume, some showed goiter (13%) or atrophic thyroiditis (8%). A minority

Table 1 Thyroid status of control subjects and patients with thyroiditis or multinodular goiter. Values are means $\pm$ s.d. for normally distributed variables.

	Control	Thyroiditis	Multinodular goiter	P
n	37	70	20	
Age (years)	46 $\pm$ 16	45 $\pm$ 13	45 $\pm$ 7	ns
Gender (%M/F)	4/33	7/63	2/18	ns
Thyroid volume	9 $\pm$ 3	11 $\pm$ 8	17 $\pm$ 8*	0.007
Hypoechoic (%)	0	66	0	0.0001
Hypervascularity (%)	0	35	0	0.0001
TSH (μ U/ml)	1.7 $\pm$ 0.8	3.8 $\pm$ 4.5 [§]	0.9 $\pm$ 0.6	0.0006
AbTPO (UI/ml)	8 $\pm$ 4	421 $\pm$ 402 ^{°^}	9 $\pm$ 6	0.0001
AbTg (UI/ml)	13 $\pm$ 8	218 $\pm$ 422 ^{°^}	21 $\pm$ 25	0.0001
AbTPO positivity (%)	0	76	0	0.0001
AbTg positivity (%)	0	60	0	0.0001
Hypothyroidism				
Serum TSH > 3.5 μ U/ml (%)	0	31	0	0.001
CXCL10 (pg/ml)	78 $\pm$ 33	147 $\pm$ 126 ^{°^}	83 $\pm$ 32	0.007
CCL2 (pg/ml)	399 $\pm$ 142	379 $\pm$ 137	389 $\pm$ 110	ns

AbTPO, Antithyroperoxidase antibody; AbTg, Antithyroglobulin antibody; TSH, Thyroid-stimulating hormone.

*P < 0.005 GMN vs ctrl; °P < 0.005 GMN vs TH; §P < 0.01 TH vs ctrl or ^GMN < 0.01. ns, not significant.

of patients (4%) were submitted to fine-needle aspiration to exclude the presence of thyroid cancer or lymphoma; in these cases, cytology confirmed the presence of a lymphocytic infiltration.

Controls

Two age- and sex-matched controls groups were used. The first control group (control I, $n = 37$) consisted of 37 subjects, extracted from a random sample of the general population from the same geographic area (33) in whom a complete thyroid work-up (history, physical examination, thyroid-stimulating hormone (TSH), FT3, FT4, anti-thyroglobulin (AbTg) and anti-thyroid peroxidase (AbTPO) antibody measurements, and ultrasonography) was available, and excluded the presence of thyroid disorders. A second control group comprised 20 patients with non-toxic multinodular goiter extracted from the same random sample of the general population. The majority of these patients had a normal thyroid volume, some showed goiter (30%). All these patients were submitted to fine-needle aspiration to exclude the presence of thyroid cancer; cytology confirmed the absence of a malignancy. In all patients and controls, a blood sample was collected in the morning, after overnight fasting, and serum was kept frozen until thyroid hormone, thyroid autoantibody, CXCL10 and CCL2 measurement.

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

Ultrasonography of the neck and fine-needle aspiration (FNA)

Neck ultrasonography was performed by the same operator, who was unaware of the results of thyroid hormone, autoantibody, CXCL10 and CCL2 measurements, using a probe (Esaote, Florence, Italy; AU5 with a sectorial 7.5 MHz transducer). Thyroid volume was calculated using the ellipsoid formula, as described (31, 34). 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) in order to evaluate structural abnormalities of thyroid tissue associated with thyroid autoimmunity (32, 34). The presence of thyroid nodules was recorded, and nodules with a diameter >10 mm were submitted to ultrasonography-guided FNA, which was performed by the same operator, using a free-hand method as already described (33, 34).

Thyroid blood flow

Thyroid blood flow (TBF) by color-flow doppler (CFD) was studied in all patients (35). The CFD pattern was defined as 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 (35). In AT patients TBF bore no relation to the thyroid status, and was type 0 in 63%, type I in 31%, type II in 6% of patients, while none had type III CFD pattern.

Laboratory evaluation

Thyroid function and thyroid autoantibodies were measured as previously described (31). Circulating FT3 and FT4 were measured by commercial RIA kits (AMERLEX-MAB FT3/FT4 Kit; Amersham Biosciences, Little Chalfont, UK). Serum TSH (DiaSorin, Saluggia, Italy), AbTPO and AbTg (ICN Pharmaceuticals, Costa Mesa, CA, USA) were evaluated by IRMA methods. For AbTg and AbTPO, positivity was set at >50 , and >10 UI/ml, respectively.

Chemokines assay

Serum CXCL10 levels were assayed by a quantitative sandwich immunoassay using a commercially available kit (R&D Systems, Minneapolis, MN, USA), with a sensitivity ranging from 0.41–4.46 pg/ml. The intra- and interassay coefficients of variation were 3.0% and 6.9% respectively. Serum CCL2 levels were also assayed by a quantitative sandwich immunoassay using a commercially available kit (R&D Systems), with a sensitivity of less than 5.0 pg/ml. The intra- and interassay coefficients of variation were 4.7% and 5.8% respectively.

Data analysis

Values are given as means $\pm$ S.D. for normally distributed variables. Mean group values were compared by using one-way analysis of variance (ANOVA) for normally distributed variables, otherwise by the Mann–Whitney U test. Proportions were compared by the χ^2 test. *Post-hoc* comparisons on normally distributed variables were carried out using the Bonferroni–Dunn test. Univariate analysis was performed by simple regression and multivariate analysis was performed by multiple linear regression analysis using CXCL10 or CCL2 as dependent variables and age, thyroid volume, TSH, FT4, AbTPO, hypoechoic pattern, and the presence of hypervascularity as covariates.

Results

The demographic and clinical features of patients and controls are reported in Table 1.

As expected, patients with AT showed significantly higher serum TSH and thyroid autoantibody levels, as well as hypoechogenicity and hypervascularity of the thyroid gland in comparison to both control groups. Interestingly, mean CXCL10 serum level was significantly higher in patients with thyroiditis than in controls or multinodular goiter patients, while serum

Table 2 Clinical and biochemical features of patients with chronic autoimmune thyroiditis with or without hypothyroidism. Values are means±s.d. for normally distributed variables.

	No hypothyroidism	Hypothyroidism	P
Age (years)	40±10	55±13	0.0001
Gender (M/F)	6/42	1/21	ns
Thyroid volume (ml)	13±9	9±6	0.09
Hypoechoic (score units)	0.6±0.5	0.9±0.3	0.01
Hypervascularity (score units)	0.3±0.5	0.5±0.5	ns
FT4 (ng/dl)	8.9±1.6	7.2±1.3	0.02
FT3 (pg/ml)	3.8±0.6	3.6±0.2	0.57
AbTPO (UI/ml)	365±397	544±392	0.08
AbTg (UI/ml)	216±459	220±331	0.97
CXCL10 (pg/ml)	125±84	197±180	0.02
CCL2 (pg/ml)	366±129	431±144	0.03

AbTPO, antithyroperoxidase antibody; AbTg, antithyroglobulin antibody; TSH, thyroid-stimulating hormone. ns, not significant.

CCL2 levels were found comparable between groups (Table 1). The assignment of AT patients to two groups on the basis of their normal or impaired thyroid function demonstrated significantly older age, lower FT4 and higher CXCL10 and CCL2 in AT patients with hypothyroidism (Table 2).

In order to better define the role of increased serum chemokines in AT, CXCL10 and CCL2 were studied separately in relation to clinical features of AT patients. As shown in Table 3, serum CXCL10 levels were significantly increased in hypothyroid patients and in those with an hypoechoic pattern ($P = 0.0004$ and $P = 0.0001$, respectively). Nearly significant differences ($P = 0.06$) were observed in relation to AbTPO positivity and age.

By contrast, serum CCL2 levels (Table 4) were significantly increased in patients older than 50 years and to a lesser degree of significance in those with hypothyroidism ($P = 0.0001$ and $P = 0.03$, respectively). Finally, no correlation between CXCL10 and CCL2 serum levels could be demonstrated.

In a multiple linear regression model including age, thyroid volume, TSH, FT4, Ab TPO, hypoechoic pattern, and the presence of hypervascularity, only TSH was significantly related to ln of serum CXCL10 levels [R.C. 0.143 C.I. (0.042–0.245); $P = 0.0059$] (Fig. 1). A similar regression model performed with

Table 4 CCL2 levels in relation to clinical features of chronic autoimmune thyroiditis subjects. Values are means±s.d.

	No	Yes	P
Age > 50	337±118	463±137	0.0001
Gender (F)	430±135	377±136	0.33
Thyroid volume < 6 ml	372±135	430±148	0.24
Thyroid volume > 20 ml	372±142	431±79	0.22
Hypoechoic	349±112	400±145	0.14
Power Doppler, hypervascularity	388±131	365±150	0.53
AbTPO positivity	377±131	380±131	0.94
AbTg positivity	166±147	385±123	0.56
Serum TSH > 3.5 µU/ml	356±129	431±144	0.03

AbTPO, antithyroperoxidase antibody; AbTg, antithyroglobulin antibody; TSH, thyroid-stimulating hormone.

CCL2 as dependent variable showed that only age was significantly related to the circulating chemokine level [R.C. 5.412 C.I. (3.838–6.986); $P < 0.0001$] (Fig. 2).

By defining a high CXCL10 level as a value at least 2 s.d. above the mean value of the control group, 26% of patients with AT, 5% of multinodular goiter patients and none of the control subjects had high CXCL10 ($P < 0.0001$). Instead, high CCL2 levels defined as above characterized 1% of patients with AT, none of the multinodular goiter patients and 3% of controls.

Table 3 CXCL10 levels in relating to clinical features of chronic autoimmune thyroiditis subjects. Values are means±s.d.

	No	Yes	P
Age > 50 years	107±56	146±148	0.06
Gender (F)	97±43	128±113	0.33
Thyroid volume < 6 ml	118±97	179±173	0.07
Thyroid volume > 20 ml	123±110	128±100	0.87
Hypoechoic	89±37	171±146	0.0001
Power Doppler, hypervascularity	115±94	153±141	0.12
AbTPO positivity	115±94	143±118	0.06
AbTg positivity	119±110	129±106	0.62
Serum TSH > 3.5 µU/ml	105±70	192±177	0.0004

AbTPO, antithyroperoxidase antibody; AbTg, antithyroglobulin antibody; TSH, thyroid-stimulating hormone.

Figure 1 In a multiple regression model including age, thyroid volume, TSH, FT4, AbTPO, hypoechoic pattern and hypervascularity, only TSH was significantly related to serum CXCL10 levels.

Figure 2 In a multiple regression model including age, thyroid volume, TSH, FT₄, AbTPO, hypoechoic pattern and hypervascularity, only age was significantly related to serum CCL2 levels.

Discussion

The AT patients in the present study were typical with respect to clinical phenotype and pattern of autoimmune markers, furthermore all patients were newly diagnosed with AT. Thyroid volume was significantly larger in patients with multinodular goiter and comparable between AT patients and controls. Circulating CXCL10 levels were clearly elevated in AT patients as compared with normal controls or patients with multinodular goiter, while comparable serum CCL2 were found between groups. In patients with AT, multivariate analysis shows that serum CXCL10 levels were associated with TSH independently from other possible confounders, while serum CCL2 were significantly associated only with age.

Within the AT group, higher CXCL10 levels were associated with a hypoechoic gland, and hypothyroidism, while in hypothyroid patients with AT, CXCL10 levels were more strongly associated with the hypothyroidism itself than with thyroid autoantibody levels or thyroid volume. Furthermore, the significantly higher CXCL10 levels in patients with an hypoechoic thyroid gland at ultrasound, a reliable sign of lymphocytic infiltration, is in line with previous reports showing that CXCL10 plays a role in massive inflammatory infiltrates in the liver (36). Such results suggest that raised CXCL10 is not only associated with the autoimmune process itself, but may be a marker of an aggressive thyroiditic process. The role of CXCL10 in autoimmune process-dependent thyroid destruction, can be reasonably assumed in view of its chemoattractant activity for Th1 lymphocytes secreting IFN- γ whose intraglandular production results in thyrocyte apoptosis (37) and severe hypothyroidism (38). CXCL10-induced recruitment of Th1 lymphocytes, which secrete interferon- γ , in turn stimulates chemokine production by follicular cells, thus maintaining the autoimmune process (6, 27).

Previous studies evaluating intraglandular mRNA expression of CC chemokines reported comparable CCL2 levels between patients with AT and multinodular goiter, in agreement with our results (6, 25). However, comparisons between data obtained by tissutal quantification of chemokine expression and assessment of circulating levels must be always careful.

A previous paper has reported increased serum CCL2 levels in AT with respect to normal controls or patients with cold nodules, with a weak but significant association between circulating chemokine level and AbTg and AbTPO in AT patients (26). In our experience, neither finding could be confirmed.

Several factors may help to explain such discrepancy. Mainly the fact that in the previous reports (26), AT patients were significantly older than controls and several reports have demonstrated that serum CCL2 levels increase with age even in normal subjects (39, 40). Differences may also reflect the fact that some patients in the study by Kokkotou *et al.* (26) were under L-T4 therapy that potentially may affect chemokine secretion; owing to the levothyroxine substitutive treatment, any comparison between euthyroid and hypothyroid AT patients could not be performed. In our serie, despite comparable CCL2 levels between HT patients and controls, significantly higher CCL2 levels were observed in hypothyroid with respect to euthyroid HT patients. However, multivariate regression analysis clearly showed that such relation was mainly due to older age of such patients, identifying age as the only factor among those taken into account being closely linked to CCL2. Furthermore, while CXCL10 was higher in patients with hypothyroidism and those with an hypoechogenic pattern, CCL2 showed no variation with respect to thyroid gland echogenicity, thus supporting the concept of a minor role played by CCL2. In addition, all of our patients were newly diagnosed AT, while in the Kokkotu *et al.* study, no information was given with regards to onset of disease, such a variable seems crucial as different time points in the disease process and is surely an important factor in determining serum concentration of markers of autoimmunity (28–30).

Besides their chemotactic activity, chemokines modulate the immune responses through the recruitment of specific T helper subsets and cytokine production. Several experimental evidences have demonstrated that *in vivo*, cytokines act in distinct combinations that determine the final effect on the target cells. The patterns of chemokines secreted by Th1 and Th2 cells constitute paradigmatic combinations that specifically drive particular types of immune response, but other such combinations are probably yet to be unveiled (25). Therefore the simultaneous assessment of different chemokines may be potentially of great interest.

In this view, relevant findings arise from two previous reports in which CXCL10 and CCL2 have been contemporarily assessed in the serum and cerebrospinal fluid

(CSF) of MS patients, showing no correlation between the two chemokines but their different behaviour in relation to the phase of disease. In details, CXCL10 was higher in acute MS and lower in stable disease while the contrary characterized CCL2 secretion profile, demonstrating a pathogenetic role for both chemokines with reciprocal changes according to the clinical phase of MS (28–30). However, no correlation between serum CXCL10 and CCL2 levels in AT patients was found in our study group. Increased CXCL10 and normal CCL2 levels in AT are consistent with the recently proposed immunoregulatory characteristics of AT. In fact, CXCL10 is clearly a Th1 oriented chemokine while CCL2 is regulated by IL-4, the cardinal Th2 cytokine, and influences T cells toward Th2 commitment. It has been proposed that an environment strongly enriched in Th1 (CXCL10) accompanied by inadequate Th2 response (CCL2) would lead to very severe non-remitting disease, as previously suggested by reports demonstrating the association of increased serum CXCL10 levels with more severe course and aggressiveness of Th1 mediated autoimmune reactions (12).

Experiments performed *in vitro* have demonstrated a differential role for RANTES (known to favour the attraction of Th1 cells) and CCL2 (preferentially active on Th2 cells) in murine experimental AT, with distinct secretion profiles for the two chemokines during the onset, the course, and the remission of disease. This demonstrates that CCL2 attracts specific immune regulatory cells that down-regulate the autoimmune reaction as shown by a decrease in murine thyreoglobulin proliferative response and in a lesser degree of thyroid infiltration (41).

The study design (only patients with newly diagnosed AT) does not permit to draw conclusion regarding the pathogenetic role of CCL2 in long standing AT, as it is possible that circulating chemokine levels at other stages of disease may be substantially different as a result of the shift in the Th1/Th2 balance during later stages of disease, acting as a feed-back mechanism widely described with disease progression in several human autoimmune conditions.

In conclusion, our results, obtained in a large cohort of newly diagnosed AT patients showed increased CXCL10 which was more evident in hypothyroid patients with a more aggressive disorder and normal CCL2 serum levels in AT. Prospective clinical trials performed in large patient cohorts will be required to evaluate the differential impact of autoimmunity and hypothyroidism 'per se', on CXCL10 serum levels, and the utility for the clinical management in AT patients.

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