Simultaneous measurement of serum chemokines in autoimmune thyroid diseases: possible role of IP-10 in the inflammatory response

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Abstract. Autoimmune thyroid diseases (AITDs), including Graves’ diseases (GD) and Hashimoto’s thyroiditis (HT), are the most common autoimmune diseases, and are mainly mediated by T cells that produce cytokines and chemokines in abnormal amounts. Few reports have described the circulating chemokines active in AITDs. Recently, we used a new multiplex immunobead assay to simultaneously measure cytokines and chemokines in small volume serum samples from patients with AITDs. We measured 23 selected serum chemokines in patients with GD (n=45) or HT (n=26), and healthy controls (n=9). GD patients were further classified as either untreated, intractable, or in remission, while HT patients were classified as either hypothyroid or euthyroid. Of the 23 serum chemokines assayed, only the serum level of IP-10 (CXCL10/interferon-γ-inducible protein 10) was elevated, depending on disease activity, in GD or HT compared with healthy controls. However, the serum level of IP-10 was also increased in both untreated GD patients and hypothyroid HT patients, suggesting that levels of this cytokine may not be affected by disease specificity. In conclusion, autoimmune inflammation in patients with AITD is closely related to the level of the serum chemokine, IP-10. Therefore, IP-10 might be a good biomarker for tissue inflammation in the thyroid, but not a useful biomarker for predicting disease specific activity, the progression of AITDs, or responsiveness to treatment because of its independence from thyroid function or disease specificity.

Key words: Chemokine, Graves’ disease, Hashimoto’s thyroiditis, IP-10, Multiplex assay
α, are also stimulated to secrete IP-10 and to amplify the autoimmune process in a feedback loop [20]. It has been reported that serum levels of IP-10 are increased in patients with GD and HT [14]. Furthermore, it has been postulated that IP-10 could be a possible marker of an aggressive inflammatory response in the thyroid, which subsequently leads to thyroid destruction and hypothyroidism [19]. Up until now, it has been unclear how chemokines interact with each other to lead to progressive autoimmune processes. Studies reporting on the simultaneous measurement of serum chemokines in AITDs are lacking. As cytokines and chemokines form a complex network, with both paracrine as well as autocrine functions, elucidation of this network has been hindered by a lack of techniques for the simultaneous analysis of multiple cytokines and chemokines. We can now simultaneously measure cytokines and chemokines in small volume samples by new multiplex immunobead assay technology.

In the present study, we investigated serum chemokine profiles in patients with AITDs by the simultaneous measurement of multiple serum chemokines in order to identify a useful biomarker for predicting disease activity and the progression of AITDs.

Materials and Methods

Subjects

The subjects of this study were outpatients at Fujita Health University Hospital. Twenty-one patients who met the criteria for untreated GD, 11 intractable GD patients, 13 GD patients in remission, 12 HT patients with hypothyroidism, 14 HT patients with euthyroidism and nine healthy controls were recruited. All untreated GD patients were newly diagnosed on the basis of clinical and laboratory examinations and positive results for anti-TSH receptor antibody (TRAb).

Intractable GD patients had been treated with more than 10 mg methimazole or 150 mg propylthiouracil for at least two years and were still positive for TRAb. GD patients in remission had been maintained in an euthyroid state without taking medication for more than two years; they were all negative for TRAb. All HT patients were diagnosed on the basis of positive laboratory results for anti-thyroglobulin antibody (TgAb) and anti-thyroperoxidase antibody (TPOAb). HT patients with hypothyroidism were newly diagnosed without taking medication. HT patients with euthyroidism received no medication. Healthy controls had no goiter or history of thyroid disease, and were negative for TRAb, TgAb and TPOAb. All participants provided written, informed consent. The study protocol was approved by the Ethics Committee of Fujita Health University School of Medicine.

Serum levels of TSH (normal level: 0.35-4.94 μIU/mL), free T3 (FT3) (normal level: 1.71-3.71 pg/mL) and free T4 (FT4) (normal level: 0.70-1.48 ng/dL) were measured using a chemiluminescent enzyme immunoassay (Ortho-Clinical Diagnostics, Raritan, NJ, USA).

Serum TRAb (normal level: < 2.0 IU/L) was measured using an electrochemiluminescent immunoassay (ECLusys TRAb, Roche Diagnostics, Tokyo, Japan). Serum TgAb (normal level: < 28 IU/mL) and TPOAb (normal level: 16 IU/mL) were measured using a radioimmunoassay (Cosmic Corporation, Tokyo, Japan).

Graves’ ophthalmopathy (GO) was defined as having exophthalmos, blepharadenoma, diplopia, or any orbital sign such as Graefe, Moebius and so on.

Chemokine assay

Serum samples for the measurement of chemokines were stored at -80°C until assay. All samples were analyzed on the same day to minimize day-to-day variation. Chemokine levels in each serum sample were detected using a MAGPIX®-Luminex® assay (MILLIPLEX® Multiplex Assays, Millipore, Tokyo, Japan) according to manufacturer’s instructions. Twenty-three different chemokines were studied: I-309 (also known as CCL1), MCP-1 (CCL2), MIP-1α (CCL3), MIP-1β (CCL4), RANTES (CCL5), MCP-3 (CCL7), MCP-2 (CCL8), EOTAXIN (CCL11), MCP4 (CCL13), MIP-1δ (CCL15), TARC (CCL17), 6Ckine (CCL21), MDC (CCL22), EOTAXIN-2 (CCL24), EOTAXIN-3 (CCL26), CTACK (CCL27), GRO (CXCL1, 2, 3), ENA-78 (CXCL5), IL-8 (CXCL8), IP-10 (CXCL10), SDF-1 (CXCL12), BCA-1 (CXCL13), and FRACTAL (CX3CL-1).

Statistical analysis

Results are expressed as mean ± standard deviation and median (25th–75th percentiles). All statistical analyses were performed using JMP ver 10.0 statistical software (SAS Institute, Cary, NC, USA). A difference between experimental groups was considered to be significant when the P value was < 5 %. Mean groups of clinical parameters were compared using unpaired Student’s t-test. Median groups of clinical parameters were compared using Wilcoxon tests. Serum chemokine levels were compared by non-parametric multiple
comparison, Wilcoxon tests. The correlation between serum chemokine levels and clinical parameters were analysed by the Pearson product-moment correlation.

Results

Clinical characteristics
The clinical characteristics of participants are shown in Table 1. A total of 80 participants, including 21 patients with untreated GD, 11 patients with intractable GD, 13 GD patients in remission, 12 HT patients with hypothyroid, 14 HT patients with euthyroidism and nine age- and sex-matched controls were recruited in this study. Patients with untreated or intractable GD had higher serum levels of FT3, FT4, TRAb, TgAb and TPOAb, and higher serum levels of TSH, than controls. HT patients had significantly higher serum levels of TgAb and TPOAb than controls. Patients with hypothyroid HT showed lower serum levels of FT3 and FT4, and higher serum levels of TSH than controls. The serum levels of FT3, FT4 and TSH in GD patients in remission and in HT patients with euthyroidism did not differ from those of controls.

Serum chemokine concentrations
The results of serum chemokine concentrations from the MAGPIX®-Luminex® assay are presented in Table 2. The serum levels of 11 chemokines (MCP-2, EOTAXIN MCP-4, MIP-1α, MDC, EOTAXINE-2, CTACK, IP-10, SDF-1, BCA-1 and FRACATL) showed some significant differences across patient groups and controls. In patients with untreated GD, the serum levels of six chemokines (MIP-1α, MCP-2, EOTAXIN-2, CTACK, IP-10, BCA-1) were significantly higher compared with healthy controls (P < 0.05 for all), the serum levels of two chemokines (MIP-1α, IP-10) were significantly increased compared with intractable GD patients (P < 0.05 for both), and the serum level of one chemokine (IP-10) was increased compared with GD patients in remission (P < 0.05). Comparing intractable GD patients to GD patients in remission, only the serum levels of one chemokine (RANTES) were significantly higher (P < 0.05).

For HT patients with hypothyroidism, the serum levels of four chemokines (MCP-4, MDC, IP-10, BCA-1) were greater and the serum level of one chemokine (SDF-1) was less than those of control (P < 0.05 for all). In HT patients with hypothyroidism, the serum level of only one chemokine (SDF-1) was less than for euthyroid HT patients (P < 0.05).

IP-10 (CXCL10)
Of all chemokines currently measured, only the serum level of IP-10 showed an increase, depending on disease activity, in GD or HT patients, compared with controls (Table 2 and Fig. 1). Among GD patients, the serum level of IP-10 in untreated GD patients was highest, although there was no difference in levels between
Table 2  Serum chemokine concentration (pg/mL)

<table>
<thead>
<tr>
<th>Chemokine</th>
<th></th>
<th>Healthy controls</th>
<th>Untreated GD</th>
<th>Intractable GD</th>
<th>Remission GD</th>
<th>Hypothyroid HT</th>
<th>Euthyroid HT</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL1</td>
<td>I-309</td>
<td>4.3 ± 9.4</td>
<td>4.9 ± 1.4</td>
<td>6.0 ± 4.8</td>
<td>9.1 ± 18.0</td>
<td>5.2 ± 2.1</td>
<td>4.7 ± 1.3</td>
</tr>
<tr>
<td>CCL2</td>
<td>MCP-1</td>
<td>380.5 ± 73.1</td>
<td>506.0 ± 173.2</td>
<td>504.0 ± 224.2</td>
<td>432.2 ± 145.0</td>
<td>447.9 ± 130.9</td>
<td>467.8 ± 207.3</td>
</tr>
<tr>
<td>CCL3</td>
<td>MIP-1α</td>
<td>23.8 ± 55.6</td>
<td>9.8 ± 5.5</td>
<td>6.2 ± 4.9</td>
<td>9.1 ± 9.4</td>
<td>15.2 ± 11.2</td>
<td>8.0 ± 6.0</td>
</tr>
<tr>
<td>CCL4</td>
<td>MIP-1β</td>
<td>28.3 ± 22.8</td>
<td>39.3 ± 20.5</td>
<td>62.9 ± 74.0</td>
<td>42.1 ± 43.9</td>
<td>93.8 ± 173.9</td>
<td>28.7 ± 23.8</td>
</tr>
<tr>
<td>CCL5</td>
<td>RANTES</td>
<td>7665 ± 5375</td>
<td>6251 ± 6627</td>
<td>9206 ± 10553</td>
<td>4464 ± 5434</td>
<td>15194 ± 15226</td>
<td>10674 ± 11232</td>
</tr>
<tr>
<td>CCL7</td>
<td>MCP-3</td>
<td>10.3 ± 13.0</td>
<td>8.1 ± 11.3</td>
<td>21.8 ± 35.0</td>
<td>11.2 ± 26.2</td>
<td>0.3 ± 116.5</td>
<td>7.1 ± 9.8</td>
</tr>
<tr>
<td>CCL8</td>
<td>MCP-2</td>
<td>26.4 ± 18.0</td>
<td>48.3 ± 21.3</td>
<td>6.2 ± 4.9</td>
<td>47.5 ± 23.9</td>
<td>42.3 ± 29.3</td>
<td>44.4 ± 21.5</td>
</tr>
<tr>
<td>CCL11</td>
<td>EOTAXIN</td>
<td>63.2 ± 21.1</td>
<td>69.3 ± 30.3</td>
<td>111.0 ± 94.6</td>
<td>68.1 ± 18.6</td>
<td>102.9 ± 71.4</td>
<td>66.4 ± 30.5</td>
</tr>
<tr>
<td>CCL13</td>
<td>MCP-4</td>
<td>33.4 ± 7.8</td>
<td>43.9 ± 14.6</td>
<td>58.8 ± 36.8</td>
<td>59.5 ± 49.5</td>
<td>51.3 ± 20.2</td>
<td>53.0 ± 25.2</td>
</tr>
<tr>
<td>CCL15</td>
<td>MIP-15</td>
<td>1203 ± 675</td>
<td>2681 ± 2405</td>
<td>1887 ± 1033</td>
<td>2586 ± 2440</td>
<td>1412 ± 788</td>
<td>1773 ± 1016</td>
</tr>
<tr>
<td>CCL17</td>
<td>TARC</td>
<td>125.8 ± 66.7</td>
<td>144.5 ± 76.6</td>
<td>170.4 ± 72.1</td>
<td>163.8 ± 81.2</td>
<td>154.5 ± 80.4</td>
<td>165.4 ± 70.1</td>
</tr>
<tr>
<td>CCL21</td>
<td>6Cine</td>
<td>186.1 ± 65.1</td>
<td>301.3 ± 173.6</td>
<td>420.4 ± 568.1</td>
<td>354.6 ± 293.8</td>
<td>305.2 ± 244.6</td>
<td>266.9 ± 194.7</td>
</tr>
<tr>
<td>CCL22</td>
<td>MDC</td>
<td>619.8 ± 22.6</td>
<td>840.2 ± 424.6</td>
<td>996.0 ± 423.7</td>
<td>783.2 ± 324.8</td>
<td>930.4 ± 314.5</td>
<td>908.4 ± 337.8</td>
</tr>
<tr>
<td>CCL24</td>
<td>EOTAXIN-2</td>
<td>451.4 ± 138.8</td>
<td>641.2 ± 223.7</td>
<td>808.4 ± 430.3</td>
<td>517.3 ± 240.0</td>
<td>462.1 ± 176.3</td>
<td>535.3 ± 228.4</td>
</tr>
<tr>
<td>CCL26</td>
<td>EOTAXIN-3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CCL27</td>
<td>CTACK</td>
<td>521.5 ± 144.0</td>
<td>871.5 ± 366.5</td>
<td>721.1 ± 245.6</td>
<td>1003.5 ± 483.7</td>
<td>614.0 ± 261.6</td>
<td>861.6 ± 545.9</td>
</tr>
<tr>
<td>CXCL1,2,3</td>
<td>GRO</td>
<td>586.3 ± 160.6</td>
<td>597.1 ± 190.4</td>
<td>778.7 ± 368.2</td>
<td>567.1 ± 147.0</td>
<td>924.8 ± 457.1</td>
<td>788.5 ± 407.1</td>
</tr>
<tr>
<td>CXCL5</td>
<td>ENA-78</td>
<td>1057 ± 543</td>
<td>1542 ± 884</td>
<td>1495 ± 703</td>
<td>1698 ± 1793</td>
<td>1308 ± 788</td>
<td>1375 ± 702</td>
</tr>
<tr>
<td>CXCL8</td>
<td>IL-8</td>
<td>11.2 ± 19.2</td>
<td>13.7 ± 9.4</td>
<td>13.9 ± 13.5</td>
<td>10.6 ± 8.5</td>
<td>17.5 ± 9.4</td>
<td>10.2 ± 9.8</td>
</tr>
<tr>
<td>CXCL10</td>
<td>IP-10</td>
<td>87.1 ± 12.9</td>
<td>204.7 ± 59.2</td>
<td>159.4 ± 73.7</td>
<td>136.8 ± 63.8</td>
<td>208.5 ± 124.3</td>
<td>151.0 ± 73.1</td>
</tr>
<tr>
<td>CXCL12</td>
<td>SDF-1</td>
<td>911.7 ± 255.0</td>
<td>883.3 ± 561.9</td>
<td>1023.6 ± 26.2</td>
<td>1133.0 ± 664.6</td>
<td>587.4 ± 329.7</td>
<td>826.7 ± 291.9</td>
</tr>
<tr>
<td>CXCL13</td>
<td>BCA-1</td>
<td>51.6 ± 19.5</td>
<td>119.9 ± 78.3</td>
<td>148.3 ± 98.5</td>
<td>120.2 ± 85.2</td>
<td>97.8 ± 48.8</td>
<td>84.9 ± 63.3</td>
</tr>
<tr>
<td>CX3CL-1</td>
<td>FRACTAL</td>
<td>54.4 ± 77.8</td>
<td>52.2 ± 57.6</td>
<td>66.7 ± 54.4</td>
<td>205.3 ± 401.9</td>
<td>515.9 ± 1499.8</td>
<td>47.1 ± 54.0</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviation. ND: not detected

*P < 0.05 vs. Healthy controls, †P < 0.05 vs. intractable GD, ‡P < 0.05 vs. remission GD, §P < 0.05 vs. euthyroid HT

Fig 1.  Serum levels of IP-10
The serum levels of IP-10 were determined in patients with untreated GD, intractable GD, GD in remission, hypothyroid HT, euthyroid HT and healthy controls.
patients with intractable GD and GD in remission. The serum level of IP-10 in patients with hypothyroid HT did not differ from that of patients with euthyroid HT. Among GD patients, the serum level of IP-10 had weak positive correlation with FT₃ \((r=0.42, P<0.05)\) and FT₄ \((r=0.43, P<0.05)\), but no correlation with TRAb. Among HT patients, the serum level of IP-10 had weak negative correlation with FT₄ \((r=0.5184, P<0.05)\), but no correlation with TSH and FT₃. The serum level of IP-10 seemed to increase in both untreated GD as well as in hypothyroid HT patients, suggesting that levels of thyroid hormone may not affect the production of IP-10. Of 45 GD patients, 15 patients (33.3%) had GO. Six of 21 patients (28.6%) were with untreated GD, 8 of 11 patients (72.7%) with intractable GD and one of 13 GD patients (7.7%) in remission, respectively. However the mean level of IP-10 was not different in patients with ophthalmopathy compared with those without it.

**Discussion**

In the present study, we simultaneously measured 23 selected serum chemokines by multiplex assay technology in patients with GD and HT, and also in healthy controls. GD patients were classified into either untreated, intractable or remission groups, while HT patients were classified into either hypothyroid or euthyroid groups. This is the first report of the simultaneous measurement of chemokines in the same serum samples, which revealed that serum levels of chemokines from GD and HT patients differed from those of healthy controls; several serum chemokines showed significant differences among various disease states of GD and HT patients.

In this study, IP-10 was the only chemokine among 23 selected chemokines to show a significant difference in serum levels compared with those of healthy controls, and all disease states of GD and HT. IP-10 plays an important role in the initiation and maintenance of inflammatory processes [13, 14, 23, 24]. IP-10 was initially identified as a chemokine that is induced by IFN-γ and that exerts its function through binding to chemokine receptor 3 (CXCR3) [25]. Recently, many reports have shown that serum and/or the tissue levels of IP-10 are increased in various autoimmune diseases such as type 1 diabetes, Graves’ ophthalmopathy, rheumatoid arthritis, systemic lupus erythematosus, mixed cryoglobulinemia, Sjogren’s syndrome, systemic sclerosis and chronic hepatitis C infection, as well as in AITD [21, 22].

Th1 T cells, which are recruited into thyroid cells and which produce IFN-γ and TNF-α, also stimulate the secretion of IP-10 and amplify the autoimmune process, leading to a feedback loop [20]. Thus, a high IP-10 serum level is a marker associated with a Th1 immune response [25]. We found significantly higher serum levels of IP-10 in all patients with GD, regardless of disease status, compared with controls. Antonelli et al. also reported increased serum levels of IP-10 in patients with GD compared with age- and sex-matched controls [26]. Our study revealed increased serum levels of IP-10 in not only patients with newly diagnosed untreated GD, but also in those with intractable GD. However, IP-10 serum levels in untreated GD patients were higher than those in intractable patients or those in remission. Therefore, the treatment may affect the level of IP-10, even if those with intractable GD or in remission GD could not reach the level of IP-10 in healthy controls.

It has been reported that serum levels of IP-10 were higher in patients with recent-onset GD than in healthy controls; in contrast, no correlation was shown between serum levels of IP-10 and clinical and biochemical factors such as sex, age, and levels of TRAb or TPOAb [27]. Additionally, Antonelli et al. reported high levels of circulating IP-10 were associated with the active phase of GD in both newly diagnosed and relapsed hyperthyroid patients; furthermore, treatment with methimazole reduced serum levels of IP-10 [28]. In support of these findings, we found that serum levels of IP-10 in patients with untreated GD patients were highest, but decreased in those in remission GD. However, there was no significant difference in IP-10 serum levels between patients with intractable GD and those in remission and postulated reasons for this include: There were not enough participants to detect small differences among patients with different durations of disease and patients of intractable GD. In addition, such groups included various types of patients, such as those who showed an initial resistance to treatment, those with a history of repeated remissions and relapses, or those showing poor adherence to medication. In both patients with hypothyroid and euthyroid HT, serum levels of IP-10 were significantly increased compared with controls. Previous reports showed that serum levels of IP-10 in patients with newly diagnosed autoimmune thyroiditis were significantly higher and that this was related to a hypoechoic ultrasonography pattern,
suggesting that IP-10 could be an inflammatory chemokine within the thyroid. [19, 29]. Our results showed a similar increase of IP-10 levels in HT patients with hypothyroidism and euthyroidism. However, no significant difference was found between them, probably because our hypothyroid group included patients with subclinical hypothyroidism.

Previous reports, to the best of our knowledge, aimed to measure serum levels of MCP-1, RANTES, TARC, MDC, IL-8, Mig and IP-10 in patients with GD, and MCP-1, MIP-1α, MIP-1β, RANTES, Mig, IP-10 and I-TAC in patients with HT [18, 26-35]. In this study, we measured for the first time serum levels of I-309, MIP-1α, MIP-1β, MCP-3, MCP-2, EOTAXIN, MCP-4, MIP-1δ, 6CKine, EOTAXIN-2, EOTAXIN-3, CTACK, GRO, ENA-78, SDF-1, BCA-1 and FRACTAL in patients with GD, and I-309, MCP-3, MCP-2, EOTAXINE, MCP-4, MIP-1δ, TARC, 6CKine, MDC, EOTAXIN-2, EOTAXIN-3, CTACK, GRO, ENA-78, IL-8, SDF-1, BCA-1 and FRACTAL in patients with HT. Of these chemokines, serum levels of MIP-1δ, MCP-2, EOTAXIN-2, CTACK and BCA-1 were increased in patients with untreated GD than in healthy controls.

Interestingly, only the serum level of RANTES was significantly different between intractable GD patients and GD patients in remission. Previous studies have shown an increased expression of RANTES in thyroid tissues and thyrocytes from AITD patients, compared with patients with non-autoimmune thyroid diseases or normal subjects [17, 36]. A recent report revealed circulating RANTES was increased in patients with GD compared to HT or nontoxic thyroid disease [35]. Another report showed that serum RANTES levels were elevated in both HT and untreated GD patients, with the latter group showing higher levels, when compared to nontoxic, multinodular goiter patients and healthy individuals; these decreased after treatment with methimazole in untreated GD patients [37]. However, since a comparison of RANTES serum levels among various disease states of GD has never been reported previously, further studies are required to determine whether this result could be useful for predicting disease activity and responsiveness to treatment in patients with GD.

The serum levels of MCP-4, MDC and BCA-1 in patients with hypothyroid HT were also increased than in healthy controls. Only the serum level of SDF-1 in patients with hypothyroidism was lower than in patients with euthyroidism. The serum level of SDF-1 did not correlate with TgAb and TPOAb (data not shown). However, since previous reports concerning circulating SDF-1 are lacking, further studies are required to clarify the role of SDF-1 in HT.

In this study, we simultaneously measured many serum chemokine levels, using multiplex immunobead assay technology, in patients with GD and HT, as well as in healthy controls. However, our study showed several limitations. Firstly, the number of recruited patients was not large enough to detect some chemokines, although a prominent increase in IP-10 was detected in relation to disease activity. Secondly, a detection limit exists on measurements by multiplex immunobead assay technology as exemplified by lack of detection of EOTAXIN-3. However, because of its advantages in time, cost, and sample savings when measuring multiple analytes simultaneously, multiplex immunobead assay is superior to the commonly used ELISA assay [38, 39].

In conclusion, we have identified a relationship between the autoimmune inflammation of patients with AITD and IP-10 by the simultaneous measurement of multiple serum chemokines. Therefore, IP-10 might be a good biomarker for tissue inflammation in thyroid, but it may not be a useful biomarker for predicting disease specific activity, the progression of AITDs or responsiveness to treatment, because this chemokine was independent of thyroid function or disease specificity. Further investigation is required to apply a possible biomarker for the tissue outside thyroid.

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Disclosure

None of the authors have any potential conflicts of interest associated with this research.
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