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Enterovirus Markers and Serum CXCL10 in Children with Type 1 Diabetes

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Abstract

Most patients with type 1 diabetes are considered to have a T-cell mediated autoimmune disease. The chemokine CXCL10 promotes the migration of activated T-cells. Virus infections might contribute to the pathogenesis of type 1 diabetes and enterovirus protein and/or genome have been detected in β -cells from a majority of tested newly diagnosed children with type 1 diabetes. The chemokine CXCL10 is induced in human islet cells by enterovirus infections *in vivo* and *in vitro*, but is not expressed in islets from normal organ donors. Since CXCL10 is a chemokine known to be induced by virus infections and/or cellular damage, our aim was to study if levels of CXCL10 is elevated in serum from children with type 1 diabetes and whether it correlates to the presence of enterovirus markers.

CXCL10, neutralizing antibody titre rises against certain enterovirus, and antibodies against GAD65 were measured in serum, and enterovirus PCR was performed on whole blood from 83 type 1 diabetes patients at onset, 48 siblings and 69 controls. CXCL10 was also measured in serum from 46 patients with proven enterovirus infection and in serum from 46 patients with other proven virus infections.

The CXCL10 serum levels were not elevated in children at onset of type 1 diabetes and there was a considerable overlap between the groups with 99 (8-498) pg/ml in serum from children with type 1 diabetes, 120 (17-538) pg/ml in serum from controls and 117 (7-448) pg/ml in siblings of the children with type 1 diabetes. The CXCL10 serum levels in patients with proven enterovirus infection were slightly increased compared to the levels in the other groups, 172 (0-585) pg/ml but there was no statistically significant difference. In contrast, CXCL10 serum levels in patients with other proven virus infections were clearly elevated 418 (34-611) pg/ml.

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3 Despite that elevated CXCL10 levels have been demonstrated in some groups of patients with
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5 type 1 diabetes, in this study the mean CXCL10 serum levels were not elevated in patients
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7 with type 1 diabetes neither in patients with proven enterovirus infection. In contrast, in
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9 patients with other virus infections the CXCL10 levels were elevated, presumably reflecting
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11 the severity or the site of infection. This suggests, that local production of CXCL10 in the
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13 affected organ cannot be measured reproducibly in serum and that its potential use in clinical
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15 practice is limited.
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For Peer Review

Introduction

Type 1 diabetes is a multifactorial disease characterized by inflammation of the pancreatic islets and immune-mediated destruction of the islet β -cells (Foulis et al., 1986). It is currently unknown what causes this immune reaction, but in addition to genetic susceptibility factors, the etiology may involve single or multiple infections with β -cell tropic viruses that could trigger a localized inflammatory response (Christen et al., 2003; Horwitz et al., 1998; Ylipaasto et al., 2004). The infiltrate in the insulitic lesions is primarily composed of IFN- γ secreting (type 1) T-lymphocytes and macrophages (Foulis et al., 1991) (Foulis, 2008). Chemokines are suspected to play an important part in mediating insulinitis due to their role as regulators of immune cell trafficking (Rossi and Zlotnik, 2000). Particularly the chemokine CXCL10, also known as interferon- γ -inducible protein (IP)-10, has been identified as a major contributor to the type 1 cellular infiltration of the islets in mouse models of diabetes (Christen et al., 2003; Li et al., 2005; Rhode et al., 2005). CXCL10 acts via a single receptor, CXCR3, which is expressed primarily on activated type 1 T-cells (Baggiolini et al., 1997). This chemokine has also been implicated in several other inflammatory and autoimmune diseases (Hasegawa et al., 2006; Nishioji et al., 2001; Wenzel et al., 2006).

Among viruses, most evidence points to the enteroviruses as environmental factors in type 1 diabetes. Markers of enterovirus infections (anti-EV-IgM, EV-RNA or titer rises of neutralizing antibodies against enterovirus) are detected more frequently in blood samples from patients with newly diagnosed type 1 diabetes than in healthy controls (Frisk et al., 1992; Frisk et al., 1985; Nairn et al., 1999; Yin et al., 2002b). In a few cases enterovirus has also been isolated from patients at the time of onset of diabetes (Cabrera-Rode et al., 2005) (Hindersson et al., 2005; Vreugdenhil et al., 2000; Yoon et al., 1979), suggesting that virus infections might play a role at the onset of type 1 diabetes. Studies using isolated human pancreatic islets have further shown that enterovirus can infect human islet cells (Elshebani et al., 2007; Frisk and Diderholm, 2000) and that its replication induces CXCL10 expression in islet cells (Berg et al., 2006), supporting the idea that these infections might precipitate immune-mediated destruction by inducing or enhancing insulinitis. A finding that strongly supports the role of enterovirus in the etiology of type 1 diabetes is the finding by Richardson et al (Richardson et al., 2009) that in 44 of 72 young type 1 diabetes patients, enterovirus protein VP1 could be detected in multiple islets. Also, in the study of Tanaka S et al (Tanaka

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3 et al., 2009) this enterovirus protein, VP1, was detected in islets from three cases of fulminant
4 type 1 diabetes but in none of the islets from healthy controls.
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9 The relationship between CXCL10 levels in serum and type 1 diabetes pathogenesis has not
10 been adequately studied in patients and no studies have examined the association between
11 serum CXCL10, enterovirus infection and the onset of type 1 diabetes. The aim of this study
12 was to measure the CXCL10 concentrations in serum from children with recent onset type 1
13 diabetes, healthy children, siblings to the type 1 diabetes children and patients with proven
14 virus infections.
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19 20 21 **Methods**

22 *Subjects*

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24 The study included 83 children under the age of 17 that were diagnosed with type 1 diabetes
25 in the county of Uppsala, Sweden, during 2000–2006, and 48 of their siblings. Blood samples
26 were obtained from these children within a week of diagnosis of the disease in the index case.
27
28 In some cases, an additional blood sample was collected from the same individuals 2–6
29 months later. The control group consisted of 69 healthy children without evidence of ongoing
30 infection, matched for age and sex to the children with type 1 diabetes. The controls were
31 recruited between one week and two years after the enrolment of the children with type 1
32 diabetes into the study. To obtain reference values of CXCL10 serum concentration during
33 virus infection, a second control group of coded serum samples from 46 subjects (adults and
34 children) diagnosed with acute enterovirus infection by serology (significant rises in
35 neutralising antibody) or virus isolation or diagnosed with other virus infections (herpes
36 simplex virus, cytomegalovirus, adenovirus or mumps virus) by serology or virus isolation
37 were included. Informed consent was given by all subjects, siblings, controls and/or their
38 parents. The study was approved by the ethics committee of the Medical Faculty of Uppsala
39 University.
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52 *Measurement of Anti-GAD65 Antibody in Serum*

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54 Anti-GAD65 antibody titers in serum from patients with type 1 diabetes, siblings and controls
55 were determined by two methods. Ninety-four samples were measured by anti-GAD65
56 radioimmunoassay (MercoDIA, Uppsala, Sweden) and 73 samples by an anti-GAD65 ELISA
57 not yet commercially available (MercoDIA, Uppsala, Sweden). To compare the two methods,
58 anti-GAD65 antibody titres were measured by both methods in of the 72 serum samples.
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Measurement of Markers of Enteroviral Infection

The presence of enterovirus RNA in mononuclear cells isolated from peripheral blood was analyzed by RT-PCR as previously described (Yin et al., 2002a). Rises in neutralising antibody titres against Coxsackie B4 virus, a member of the enterovirus group B, were detected by neutralization assays using several Coxsackie B 4 virus strains, as described previously (Frisk and Tuvemo, 2004). Briefly, serum samples from the patients with type 1 diabetes or their siblings were diluted in cell culture medium in two-fold steps from 1/20 to 1/2560 and 5 µl of each dilution were incubated with virus (100 x the 50% tissue culture infectious dose) for 90 minutes at 37°C. After the incubation, the “virus-and-serum” mixtures were transferred to green monkey kidney cell cultures in 96-well plates. The highest serum dilution able to neutralize 50% of the virus-induced cytopathic effects was recorded as the titer of neutralizing antibody. Acute and convalescent sera obtained at the time of diagnosis of type 1 diabetes and 2–6 months later respectively were compared. A four-fold or higher increase in neutralizing antibody titre between the two samples was considered significant. Positivity for enterovirus-RNA in peripheral mononuclear cells by RT-PCR or a significant rise in neutralizing antibody titers was regarded markers of a recent or ongoing EV infection. RT-PCR was performed on samples from 36 subjects (15 patients with type 1 diabetes, 11 siblings and 10 controls) and neutralizing antibody test on sera from 15 subjects (11 patients with type 1 diabetes and 4 siblings).

Measurement of Serum CXCL10/IP-10

A commercially available ELISA, Human IP-10 Cytoscreen (Biosource, Nivelles, Belgium) with a lowest detection limit of <2.0 pg/ml was used to measure human CXCL10 concentrations in the serum samples. previously

Statistical Analysis

Statistical analysis was performed using SPSS package for Windows, version 12.0.1. Paired samples were compared using Wilcoxon signed ranks test. Mann-Whitney test was used to compare two groups of unpaired samples and Kruskal-Wallis test was used to compare three or more groups of unpaired samples. A p-value lower than 0.05 was considered statistically significant.

Results

Serum CXCL10 Concentrations in Newly Diagnosed Patients with Type 1 Diabetes, their Siblings, and Healthy Controls

In Table 1 it can be seen that the median CXCL10 concentrations in serum from patients with type 1 diabetes (less than a week after diagnosis) (n=83), healthy controls (n=69), and non-diabetic siblings of the patients with type 1 diabetes (n=48). The serum concentrations of CXCL10 tended to be slightly lower in newly diagnosed patients with type 1 diabetes than in the healthy controls, but this difference did not reach statistical significance (0.079).

It has previously been suggested that female patients with newly diagnosed patients type 1 diabetes have higher CXCL10 concentrations than male patients (Rotondi et al., 2003), but no such relationship was found in our data (Table 1). There was no correlation between CXCL10 concentration in serum and the age of the subject (data not shown) or the month/season the sample was taken. The median (range) CXCL10 concentration was 103 pg/ml (8–724) n=81 in the samples taken during Spring/Summer (Mar–Aug) and 118 pg/ml (8–737) n=121 in samples taken during Autumn/Winter (Sept–Feb).

Table 1. CXCL10 serum concentrations and mean ages of the children with type 1 diabetes, their siblings, and healthy controls.

Subject Groups	n	Age ^a (years)	CXCL10 ^b (pg/ml)
Children with type 1 diabetes	83	9.5 (1.6–16.4)	99 (8–498)
Males	47	9.8 (1.6–16.4)	99 (8–448)
Females	36	9.3 (3.5–14.6)	104 (9–498)
Healthy controls	69	10.0 (1.6–17.2)	120 (17–538)
Males	40	10.3 (1.6–17.2)	117 (32–538)
Females	29	9.6 (3.8–14.8)	128 (17–442)
Siblings of the children with type 1 diabetes	48	12.4 (5.8–20.7)	117 (8–724)
Males	30	13.1 (5.8–20.7)	128 (8–724)

Females	18	11.7 (5.9–18.7)	106 (15–447)
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^a Mean (range)

^b Median (range)

CXCL10 and Enterovirus Positivity by RT-PCR or Serology

The children with type 1 diabetes, their siblings and healthy control subjects were divided into two groups based on the presence or absence of markers of enterovirus infection in the circulation. Enterovirus positivity was defined as the detection of enterovirus-RNA in peripheral blood mononuclear cells by RT-PCR and/or a significant rise in neutralizing antibody titers against enterovirus between the acute and convalescent serum sample (positive by serology), the latter only in samples from children with type 1 diabetes and their siblings. No correlations between the CXCL10 concentrations in sera from subjects positive or negative for markers of enterovirus infection (Table 2) could be found.

Table 2. CXCL10 concentration in subjects positive or negative for markers of enterovirus infection.

Subject groups	n	CXCL10 ^a (pg/ml)
EV-positive by serology and/or RT-PCR	24	103 (60–401)
EV-negative by serology and RT-PCR	12	99 (37–210)

^a Median (range)

To obtain reference values of CXCL10 serum concentrations during enterovirus infection, sera were obtained from non-diabetic subjects (adults and children) diagnosed with acute enterovirus infection by virus isolation (n=9) or serology (n=16) and from subjects diagnosed with other virus infections (herpes simplex virus, cytomegalovirus, adenovirus, or parotitis virus) by serology (n=9) or virus isolation (n=4). The serum CXCL10 concentrations were significantly higher, $p > 0.023$, in subjects with non-enterovirus infections than in subjects with enterovirus infection (Mann-Whitney U-test, $p < 0.05$) (Table 3).

Table 3. Serum CXCL10 concentration in subjects diagnosed with acute virus infection.

Subject groups	n	CXCL10 ^a (pg/ml)
Acute enterovirus infection	25	172 (0–585)
Infection with other viruses (herpes simplex virus, cytomegalovirus, adenovirus or parotitis virus)	13	419* (34–611)

^a Median (range)

* A statistically significant difference $p < 0.05$ ($p > 0.028$) compared to the enterovirus positive group

CXCL10 and Positivity for Anti-GAD65 Antibodies

Anti-GAD65 auto-antibodies were measured by two different methods, ELISA and RIA, in 72 samples. A larger number of samples were anti-GAD positive (>10 U/ml) when measured with the ELISA method ($n=69$) than with the RIA ($n=38$), which might reflect the detection of IgM as well as IgG by the ELISA. Anti-GAD65-IgM is frequently detected in the serum of healthy children and does not predict progression to type 1 diabetes (Hoppu et al., 2004a; Hoppu et al., 2004b). However, since the presence of anti-GAD65 IgM might represent a recent seroconversion event, the CXCL10 concentrations were compared between the 38 samples that were positive in ELISA but negative in RIA and those that were positive only with the RIA ($n=31$). No differences were found in the serum levels of CXCL10 between these two groups (median concentrations 102 pg/mL and 99 pg/mL, respectively). The anti-GAD65 antibody titers measured by either RIA or ELISA, and the CXCL10 concentrations in the sera were also compared, but no correlations were found (data not shown).

Discussion

The main findings in the present study are that serum levels of CXCL10 are not increased in children at onset of type 1 diabetes compared to siblings and to healthy control children, and that no elevated serum levels of CXCL10 could be detected in patients with proven enterovirus infection. In sera from patients with other proven virus infection the CXCL10 levels were clearly increased.

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3 Three groups have earlier reported elevated serum levels of CXCL10, in adult patients with
4 type 1 diabetes (Nicoletti et al., 2002; Shimada et al., 2001), or in children with type 1
5 diabetes (Antonelli et al., 2008), at onset. In a letter Rotondi et al (Rotondi et al., 2003)
6 commented on the results from Nicoletti et al (Nicoletti et al., 2002) and also showed that,
7 when they analyzed CXCL10 levels in serum from 70 patients with type 1 diabetes and in
8 serum from 35 healthy controls the CXCL10 levels were not elevated in the former group.
9 This is in line with our finding. The reason for this discrepancy regarding the CXCL10 levels
10 is not known, but also in the study of Antonelli et al (Antonelli et al., 2008) there was a
11 considerable overlap in CXCL10 serum levels between the three groups studied. The mean
12 CXCL10 levels in serum also differed considerably between the studies. In the study of
13 Antonelli et al (Antonelli et al., 2008), the mean CXCL10 level in the group with type 1
14 diabetes was 191 ± 142 (children), in the study of Rotondi et al (Rotondi et al., 2003) it was
15 70.3 (adults), in the study of Shimada (Shimada et al., 2001) it was 166.1 (adults), and in this
16 present study it was 99 pg/mL. The corresponding figures in the healthy control group were
17 82 ± 33 , 76.6 , 41.5 and 120 pg/mL, suggesting a great variation in serum CXCL10 levels. The
18 lower levels among adult healthy controls could reflect that this group encounters fewer
19 infections. The CXCL10 levels in serum could in the present study were not affected
20 differently by factors as storage or freeze thawing of samples, since the samples have been
21 stored in the same freezer. Test of the freeze thawing on CXCL10 levels was performed and
22 no such affect on could be found (not shown).
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40 Several roles have been attributed to the chemokine CXCL10, such as chemoattraction of
41 monocytes/macrophages, T cells, NK cells, and dendritic cells as well as promotion of T cell
42 adhesion to endothelial cells. This means that cells with the appropriate receptor will migrate
43 to the source of chemokine production and release. In the case of CXCL10, cells expressing
44 the chemokine receptor CXCR3, will migrate. Chemokines such as CXCL10 are produced in
45 response to virus infections or agents that cause physical damage to a tissue. In type 1
46 diabetes the organ that most likely secretes CXCL10 in order to attract T-cells is the
47 pancreatic islets of Langerhans. This also means that the CXCL10 levels locally in the organ
48 must be higher than in the surrounding tissue and serum in order to establish a CXCL10
49 gradient (Christen et al., 2004).
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60 Most patients with type 1 diabetes are considered to have a T-cell mediated autoimmune
disease. However, a number of environmental exposures have been proposed to contribute to

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3 or trigger development of type 1 diabetes. Enterovirus remains the prime candidate by nature
4 of its tropism for β -cells and studies have shown a relationship between enterovirus and the
5 appearance of islet autoantibodies as well as a seasonal fluctuation of onset of the disease. A
6 clear relationship between enterovirus infections and type 1 diabetes has recently been
7 demonstrated in a study showing detection of the enterovirus structural protein VP1 in
8 multiple islets from 44 out of 72 young patients with recent-onset type 1 diabetes compared to
9 in only 3 of 40 in pediatric controls. In addition the enterovirus staining was restricted to the
10 insulin containing β -cells (Richardson et al., 2009). Even though the presence of virus within
11 islets of Langerhans in patients with type 1 diabetes is not enough to state that the infection
12 caused the disease such findings strongly implies that these viruses are involved in the
13 etiology of type 1 diabetes in at least a proportion of cases.
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16 In addition, it has been shown that CXCL10 is not normally secreted from isolated human
17 islets but after infection *in vitro* with enterovirus (Berg et al., 2006) it is secreted as long as
18 the virus replicates in the β -cells. A study from Tanaka S et al (Tanaka et al., 2009) showed
19 that enterovirus could be detected in the islets in pancreatic biopsies from patients at onset of
20 type 1 diabetes, and in addition this study also showed that islets positive for enterovirus also
21 stained positive for CXCL10. Enterovirus infected islet cells in patients at onset of type 1
22 diabetes also seem to secrete this chemokine, which would explain why, in these patients,
23 insulinitis can be seen in many islets and also that the composition of this insulinitis is
24 macrophages and CD8 positive T cells (Foulis, 2008; Foulis et al., 1991; Tanaka et al., 2009)
25 expressing the receptor for CXCL10.
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28 The present study is the first to investigate the levels of CXCL10 in serum at the onset of type
29 1 diabetes in children and in addition, the association between these levels and the presence of
30 markers for enterovirus infection. Despite a few earlier findings showing that elevated
31 CXCL10 levels could be detected in serum from patients at onset of type 1 diabetes, this
32 could not be confirmed in this study. Even though there exists a clear cause relationship
33 between enterovirus infection of human islets of Langerhans and secretion of CXCL10 from
34 infected cells *in vitro* and *in vivo*, it is not possible to measure elevated levels of this
35 chemokine in serum from such patients.
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37
38 It has been shown in other studies that infections with various viruses will cause elevated
39 levels of CXCL10 in serum. The present study also included serum from patients with other
40 virus infections, proven by virus isolation. The analyses of sera from these patients revealed
41 that in most of the serum samples elevated levels of CXCL10 could be detected. This was
42 however not the case in serum from patients where enterovirus was isolated. It has been
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3 suggested in most of the cases with elevated CXCL10 serum levels due to virus infection, the
4 levels correlates, to the severity and the outcome of the infection (Lee Y.R 2008).

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6 One likely explanation for our finding that the CXCL10 levels were not increased in serum
7 from patients with type 1 diabetes, patients with type 1 diabetes with markers of an ongoing
8 enterovirus infection, or patients with proven enterovirus infection without diabetes
9 (isolation), could be that the infections were severe enough.

10
11 To conclude, in the present study no elevated levels of CXCL10 in serum from patients with
12 type 1 diabetes could be detected, not even in serum from children with onset of type 1
13 diabetes and a proven enterovirus infection. In serum from patients with other virus infection
14 the CXCL10 levels were elevated as expected. It is not unlikely that the CXCL10 levels are
15 increased in the infected organ(s) despite the failure to detect it in serum and that this locally
16 increased CXCL10 level attracts immune cells such as macrophages and CD8 positive T-
17 cells. This study also shows that serum levels of CXCL10 cannot be used as a marker for type
18 1 diabetes, not even for a subgroup of type 1 diabetes where enterovirus is thought to play a
19 role in the etiology.
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34 **Acknowledgement**

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