Association between interleukin-6 polymorphism and age-at-onset of type 1 diabetes. Epistatic influences of the tumor necrosis factor-α and interleukin-1β polymorphisms

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ABSTRACT. Multiple immune mediators have been mentioned as playing a role in the pathomechanism of type1 DM. Interleukin (IL)-1β, and tumor necrosis factor (TNF)-α play a central role in the autoimmune destruction of pancreatic β-cells, whereas IL-6 inhibits TNF-α secretion, and may have some protecting effects. In our study, we aimed to investigate the association between these three cytokines' single nucleotide polymorphisms (*IL-6* gene G(-174)C, *TNF-α* gene G(-308)A and *IL-1β* gene C(3954)T polymorphisms) and age-at-onset of type 1 diabetes mellitus (T1DM) in 165 diabetic children (median age: 17 years). Polymorphisms were determined using the PCR-RFLP method. We found that the age-at-onset of T1DM was significantly different in patients with a different *IL-6* genotype (median age-at-onset of T1DM was: 8, 6 and 4.5 years in children with the (-174)GG, GC and CC genotypes, respectively; $p < 0.01$). Adjusted for *TNF-α* and *IL-1β* polymorphisms, patients with a *IL-6* (-174)CC genotype have a 3.0-fold (95% CI: 1.2-7.1) increased risk of developing diabetes before the age of 6 years than (-174)G allele carrier patients. However, we found this association to be present only in patients who carried the *TNF-α* (-308)A or *IL-1β* (3954)T allele, *i.e.* in patients with high TNF-α and high IL-1β producer genotypes. We suppose that in the case of high TNF-α and IL-1β producer genotypes, elevated proinflammatory cytokine levels result in a higher production of IL-6 in (-174)G allele carrier patients. This elevated IL-6 level may have a protective effect against the development of T1DM and may delay the destruction of pancreatic β-cells.

Keywords: age-at-onset, interleukin-1β, interleukin-6, polymorphism, tumor necrosis factor-α, type 1 diabetes mellitus

Type 1 diabetes mellitus (T1DM) is a multifactorial, autoimmune disease characterized by the destruction of β-cells, with both environmental and genetic factors contributing to the development of the disease. The events that trigger and maintain the local autoimmune process remain elusive. However, the importance of local inflammatory processes has been established [1]. Multiple immune mediators are implicated in the destruction or protection of the pancreatic β-cells including tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6.

TNF-α and IL-1β are pro-inflammatory cytokines produced primarily by activated macrophages that infiltrate the islets during the pathogenesis of diabetes [2, 3] and play a central role in β-cell destruction [4, 5]. Both TNF-α and IL-1β stimulate interleukin (IL)-6 expression [6]. In transgenic, non-diabetes-prone mice, islet overexpression of IL-6 produces a complex, localized host response. IL-6 may contribute not only to inflammatory processes that occur in autoimmune diabetes, but also to cellular neogenesis, which may indicate a role in tissue repair [7].

Higher serum levels of TNF-α and IL-6 were found in newly diagnosed T1DM children compared to cases with longer standing DM and nondiabetic controls [8]. IL-6 was also shown to be present in the islets of recently diagnosed patients with T1DM who died shortly after diagnosis of the disease [9].

Cytokines playing a role in local inflammatory processes may influence the pace of β-cell destruction and this way the onset age of diabetes. Transgenic expression of IL-6 by β-cells in mice generated on the NOD (non-obese diabetic) background promoted insulitis but delayed the onset of diabetes [10].

Single nucleotide polymorphisms (SNPs) of *IL-6*, *TNF-α* and *IL-1β* may influence the expression of the coded cytok-

Table 1
Clinical characteristics of the study population

n (boys/girls)	165 (84/81)
Median age–years (25 and 75 percentiles)	17 (14 and 19)
Median age at diagnosis – years (25 and 75 percentiles)	6 (min:1, max:15)
Median duration of diabetes – years (25 and 75 percentiles)	9 (min: 3, max:19)
Insulin dose (U/kg/day)*	0.83 ± 0.26
HbA_{1c} (%)*	8.00 ± 1.07
Weight SDS*	0.11 ± 0.86
Height SDS*	0 ± 1.12
BMI (kg/m^2) SDS*	0.23 ± 0.97
Total cholesterol (mmol/l)*	4.91 ± 1.31
Triglycerides (mmol/l)*	1.43 ± 1.02
Creatinine (µmol/l)*	83.15 ± 18.07

*Data are means ± SD. SDS: standard deviation score.

ine and in this way these candidate genes may play a role in the pathomechanism of T1DM. The interaction of these SNPs can result not only in the development of T1DM, but in a delayed onset of diabetes as well. In our study, we aimed to determine the genotype association of *IL-6* G(-174)C (IL-6 production: C < G), *TNF-α* G(-308)A (TNF-α production: G < A) and *IL-1β* C(3954)T (IL-1β production: C < T) SNPs and the clinical characteristics (age-at-onset, insulin dose, HbA_1C level, weight, height, body mass index, serum total cholesterol, triglyceride and creatinine levels) of T1DM in children.

METHODS

Blood samples were taken from 165 children with T1DM (84 boys and 81 girls), treated in the First Department of Paediatrics, Semmelweis University, Budapest. *Table 1* shows the clinical characteristics of the population studied. Total genomic DNA was extracted from whole blood samples using the method of Miller *et al*. [11]. *IL-6* G(-174)C, *TNFα* G(-308)A and *IL-1β* C(3954)T SNPs were determined by PCR and RFLP methods as previously described [12-14]. The study was approved by the Institutional Ethics Committee (6008/26/ETT/2001), informed consent was obtained from the subjects and/or from their parents.

Statistical analysis was performed using the SPSS software, version 11.5 (Chicago, IL, USA). The Kruskal-Wallis test was used for the comparison of age-at-onset of T1DM between patients with different cytokine genotypes. Dunn's multiple comparison test was used as a *post hoc* test. Normally distributed variables (insulin dose, HbA_1C level, weight SDS (standard deviation score), height SDS, BMI (body mass index) SDS, serum total cholesterol, triglyceride and creatinine levels) of patients with different genotypes were compared using the T-test for independent samples.
We divided T1DM patients into two different groups on the basis of their age at the onset of the disease (< or ≥ 6 years, because the median age-at-onset was 6 years) and multiple logistic regression analyses were performed to assess the independent association of IL-6, with the age-at-onset of T1DM. Gender and *TNF-α* and *IL-1β* genotypes were included in the model. In addition, by the using the same type of statistical analysis interaction as used for the IL-6, the other two cytokine polymorphisms were also determined. The level of statistical significance was set at $p < 0.05$.

RESULTS

Table 2 shows *TNF-α*, *IL-1β* and *IL-6* genotype distribution in our T1DM population. *TNF-α* and *IL-6* SNPs fulfilled the Hardy-Weinberg criteria, but the *IL-1β* polymorphism differed significantly from these criteria in the T1DM population studied. Insulin dose, HbA_1C level, weight SDS (standard deviation score), height SDS, BMI (body mass index) SDS, serum total cholesterol, triglyceride and creatinine level were associated with none of the polymorphisms studied (data not shown)
We found a significant difference in the age-at-onset of T1DM in patients with different *IL-6* genotypes *(table 2)*, but the onset age was not associated with *TNF-α* and *IL-1β* genotypes. Patients were divided into two groups based on their age-at-onset of diabetes (early onset: < 6 years of age and late onset disease: > 6 years of age), because the median age-at-onset was 6 years. It turned out that early onset T1DM occurred frequently (19/28 (67.8%)) among patients with the *IL-6* (-174)CC genotype, whereas only 23/65 (35.4%) of the patients carrying only the G allele experienced early onset disease *(table 3)*. We calculated the odds ratio for having early onset T1DM for the patients

Table 2
Genotype distribution of *TNF-α, IL-1β* and *IL-6* polymorphisms and the median (and 25 and 75 percentiles) age-at-onset of the disease in different genotypes. IL-6 genotype distribution in early (< 6 years) and later (≥ 6 years) onset T1DM groups

***TNFα* G(-308)A**	**(-308)GG**	**(-308)GA**	**(-308)AA**	**(-308)A allele**	**p**
T1DM *n* = 165 (%)	89 (54)	69 (42)	7 (4)	0.25	
Median age-at-onset of T1DM - year (25 and 75 percentiles)	6 (4 and 10)	6 (3 and 9.5)	6 (5 and 11)		N.S.
***IL-1β* C(3954)T**	**(3954)CC**	**(3954)CT**	**(3954)TT**	**(3954)T allele**	**p**
T1DM *n* = 165 (%)	73 (44)	82 (50)	10 (6)	0.31	
Median age-at-onset of T1DM - year (25 and 75 percentiles)	7 (3 and 10)	5 (4 and 9.5)	7 (3.7 and 10.2)		N.S.
***IL-6* genotype**	**(-174)CC**	**(-174)CG**	**(-174)GG**	**(-174)C allele**	**p**
T1DM n = 165 (%)	28 (17)	72 (44)	65 (39)	0.38	
Median age-at-onset of T1DM - year (25 and 75 percentiles)	4.5 (2 and 7)	6 (4 and 10)	8 (4 and 10)		< 0.01*

*Difference between the (-174)CC and (-174)GG genotypes.

Table 3
Frequency of early* onset (< 6 years old) and late** onset (> 6 years old) disease among 165 T1DM patients with different *IL-6* genotypes

***IL-6* genotype**	**(-174)CC**	**(-174)CG**	**(-174)GG**	**All patients**
		Number (%) of patients		
Early onset disease	19 (24.7)	35 (45.5)	23 (29.9)	77 (100.0)
Late onset disease	9 (10.2)	37 (42.0)	42 (47.7)	88 (100.0)
P value (χ^2 test)		0.014		

Table 4
Results of the logistic regression analysis of the association between the *IL-6* (-174)CC genotype and the age at onset of the disease, adjusted for gender, as well as TNF-α and IL-1β genotype IL-6 polymorphism

Carrier state	**Odds ratio* (95% confidence interval)**	**P value**
Gender girls *versus* boys	1.273 (0.674–2.402)	0.456
IL-1β (3954)TT or TC *versus* CC genotype	1.403 (0.737–2.671)	0.303
TNFα (-308)AA or GA *versus* GG genotype	0.948 (0.497–1.808)	0.871
IL-6 (-174)CC or GC *versus* GG genotype	3.034 (1.262–7.299)	0.013

* Odds ratio for having diabetes before 6 years of age.

Table 5
Association between the *IL-6* (-174)CC genotype carrier state and the age-at-onset of T1DM in different subgroups of patients as calculated by gender-adjusted multiple regression analysis. Subgroups were created on the basis of *TNF-α* and *IL-1β* genotypes

Subgroups- carriers of the:	**Odds ratio* (95% confidence interval)**	**P value**
IL-1β (3954)CC genotype	1.994 (0.571–6.958)	0.279
IL-1β (3954)TC or TT genotype	5.230 (1.351–20.238)	0.017
TNF-α (-308)GG genotype	2.437 (0.647–9.186)	0.188
TNF-α (–308)GA or AA genotype	3.540 (1.100–13.341)	0.034

* Odds ratio of the carriers versus non-carriers for having diabetes before 6 years of age.

with the *IL-6* (-174)CC genotype as compared to those with the GG or GC genotype. The unadjusted odds ratio was found to be 2.875 (1.214-6.812), p = 0.016. Next we adjusted this association to the gender of the patients as well as for *TNF-α* and *IL-1β* polymorphisms using an adjusted multiple regression analysis *(table 4)*. Patients with the *IL-6* (-174) CC genotype had more than a 3.0-fold (95% CI: 1.26-7.30) increased risk of developing diabetes before the age of 6 years, whereas no significant association with gender and the other two cytokine polymorphisms was found *(table 4)*.

Since at the gender-adjusted, multiple logistic regression analysis we found a significant interaction for the association with the onset of disease between the *IL-6* -174 SNP and the *TNF-α* -308 SNP, and the *IL-6* -174 SNP and the *IL-1β* 3954 SNP (p = 0.021 and p = 0.006, respectively), we also investigated the presence of the association between IL-6 SNP and the age-at-onset of T1DM in different subgroups: low ((-308)GG) and high ((-308)A allele carrier state) TNF-α producer, and low ((3954)CC) and high ((3954)T allele carrier state) IL-1β producer genotypes *(table 5)*. We found that the association between *IL-6* polymorphism and the age-at-onset of T1DM could only be detected in patients with a high IL-1β and high TNF-α producer genotype. In the *IL-1β* (3954)T allele carriers and in *TNF-α* (-308)A allele carrier patients, the presence of the *IL-6* (-174)CC genotype has a 5.2-fold (95% CI: 1.3-20) and a 3.4-fold (95% CI: 1.09-11.23) increased risk of developing diabetes before the age of 6 years than *IL-6* (-174)G allele carrier patients, respectively.

DISCUSSION

In our study, we investigated the association between the age-at-onset of T1DM and *TNF-α* G(-308)A, *IL-1β* C(3954)T and *IL-6* G(-174)C polymorphisms in children. Our results indicate that the *IL-6* (-174)CC genotype carrier state is associated with a younger age-at-onset, whereas the *IL-6* (-174)G allele carrier state is associated with older age-at-onset of T1DM, but only in the presence of high *IL-1β* and *TNF-α* producer genotypes.

IL-6 is known to be involved in both the amplification of and protection against inflammatory reactions [15, 16]. It is a pleiotropic cytokine expressed by a wide variety of cells: T and B cells, macrophages, dendritic cells, smooth muscle cells, fibroblasts, endothelial and pancreatic β-cells. IL-6 is released in response to infection, burns, trauma, and neoplasia, and its functions range from key roles in acute-phase protein induction to B- and T- cell growth and differentiation. IL-6 can have direct effects on cells, mediate the effects of other cytokines, be coagonistic or antagonistic in conjunction with other cytokines, and interact with glucocorticoids. The main effect of IL-6 depends on the place where it is produced, on the cell type which produce it, and also on the receptor which mediates its biological activities. As far as systemic effects are concerned, IL-6 is the major initiator of the acute phase response by hepatocytes, and a primary determinant of hepatic CRP production [17]. It has also been found to play an important role in atherosclerosis and myocardial infarction [18].

However, in the development of T1DM, IL-6 supposedly acts locally and has mostly β-cell-protecting effects [19, 20]. The higher serum levels of IL-6 found in newly diagnosed T1DM individuals [21] do not conflict with its protective role in the pathomechanism of T1DM, because these higher serum IL-6 levels may be induced by the hyperglycemia [22] that accompanies the manifestation of T1DM. This hyperglicaemia has nothing to do with the autoimmune process leading to the β-cell destruction. Higher IL-6 production may also be induced by both TNF-α or IL-1β [23-25]. Lo *et al*. [26] found a significantly positive correlation between TNF-α and IL-6 serum levels in children with T1DM. Higher serum levels of TNF-α and IL-6 were also found in newly diagnosed T1DM children compared to cases with longer standing DM and nondiabetic controls [27]. These higher IL-6 levels were measured at the time of diagnosis, but there are no data available regarding IL-6 levels at the time when the autoimmune process had begun. So the higher serum levels of IL-6 might also be the result of the metabolic state, and not the main cause of the autoimmune process. In addition, it is likely enough (however it cannot be measured in human) that serum levels of IL-6 in type 1 diabetic individuals are not indicative of the local IL-6 levels found in the islets of Langerhans.

Regarding the association between the *IL-6* G(-174)C polymorphism and T1DM, conflicting results are available. Recently, a case-control study of type 1 diabetics in the UK demonstrated a higher prevalence of the *IL-6* (-174)GG genotype in Caucasoid T1DM patients than in the control population [28]. In contrast, Kristiansen *et al*. [29] found in 253 Danish T1DM families that the *IL-6* (-174)C allele was associated with T1DM, but only in females and they also demonstrated, in accordance with Gillespie *et al*. [30], that the *IL-6* (-174)CC genotype was associated with younger age-at-onset of T1DM in females, but not in males. In these studies [29, 30], the average age-at-onset of diabetes was more than 10 years: the authors attributed this different effect of IL-6 on the pathomechanism of T1DM in males and females to the stimulated activity of the IL-6 gene by estrogen. Estrogen plays a central role in puberty in females, and is switched on from ± 9 years onwards. As in our study population, the median age at the onset of diabetes was 6 years, we cannot expect to see the difference caused by estrogen.

We demonstrated that the *IL-6* (-174)G carrier state is associated with older age-at-onset of T1DM, only in the presence of high cytokine producer *IL-1β* ((3954)T carriers) and *TNF-α* ((-308)A carriers) genotypes. Both IL-1β and TNF-α are proinflammatory cytokines, they both play a central role in the pathogenesis of T1DM, and they stimulate IL-6 expression [31]. In patients with the *IL-6* (-174)G allele, high *IL-1β* or *TNF-α* producer genotypes can enhance IL-6 production, and the IL-6 can exert its diabetes-protective effect. In accordance with this hypothesis, previous studies have found that local production of human IL-6 retards the onset of insulin-dependent diabetes mellitus in non-obese diabetic mice [32], and that IL-6 protects β-cells from inflammatory cytokine-induced cell death and functional impairment both *in vitro* and *in vivo* [33].

We found no association between *TNF-α* or *IL-1β* SNPs and the age-at-onset of the disease. However, in accordance with previous studies, the prevalence of the high *TNF-α* producer and the high *IL-1β* producer genotypes were increased in the T1DM population, compared to the Hungarian healthy reference values [34, 35].

In conclusion, our results indicate that the (-174)G carrier state of the *IL-6* gene is associated with older age-at-onset of T1DM, in the presence of high cytokine producer *IL-1β* and *TNF-α* genotypes. We suppose that in these cases, higher IL-6 production associated with the (-174)G allele in Langerhans islets, might have a protective effect against the autoimmune process and might delay the destruction of the β-cells.

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