CC-chemokine receptor CCR5-del32 mutation as a modifying pathogenetic factor in type I diabetes

Ingrid Kaleva,*, Kersti Oselinb, Piret Pärlstac, Mihkel Zilmerc, Tarvo Rajasald, Toomas Poda, Aavo-Valdur Mikelsaara

aDepartment of Human Biology and Genetics, Institute of General and Molecular Pathology, Tartu University,
Ravila Street 19, 51014 Tartu, Estonia
bInstitute of Pharmacology, Tartu University, Tartu, Estonia
cInstitute of Biochemistry, Tartu University, Tartu, Estonia
dInternal Medicine Clinic of Tartu University Clinics, Tartu, Estonia

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Abstract

The purpose of this study was to determine the CCR5-del32 allele frequency in type I (insulin-dependent) and type II (noninsulin-dependent) diabetes patients, and to test whether and how this mutation is associated with both types of diabetes. Thirty-eight type I diabetes and 111 type II diabetes patients’ genotyping was performed by polymerase chain reaction assay, and amplified products were digested with restriction enzyme EcoRI. The results were analyzed using statistical methods. No statistical differences were found in CCR5-del32 allele frequencies in types I and II diabetes patients compared with the control group of native Estonians. However, an association exists between CCR5 gene polymorphism and the clinical course of type I diabetes. In the case of wild-type CCR5, the disease starts at an earlier age. In type II diabetes, there was a difference between genotypes in morbidity to concomitant diseases, being higher in the CCR5 wild-type genotype. © 2003 Elsevier Inc. All rights reserved.

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1. Introduction

In mammals, the most important facilitators of the leukocyte maturation, migration and activation are chemoattractant cytokines—chemokines. They are involved in inflammatory and autoimmune diseases, angiogenesis or angiostasis, host response to injury, insult, and infection (Gerard & Rollins, 2001).

Chemokines mediate their activity through a large seven transmembrane-spanning G protein-coupled (G-inhibitory) receptors family. Nine genes of human receptors, CCR1 through CCR9, have been cloned. The CC-chemokine receptor genes’ cluster is located on human chromosome 3p21–24. CCR5 is a receptor for the CC-chemokines RANTES (regulated upon activation normal T cell expressed and secreted), macrophage inflammatory proteins MIP-1α and MIP-1β and MCP-2 (monocyte chemoattractant protein 2) (Samson et al., 1996). The expression of some chemokine receptors depends on the state of activation and/or differentiation of T cells. CCR5 is expressed on activated Th1 lymphocytes, monocytes, macrophages, dendritic cells, microglial cells, endothelial, and smooth muscle cells (Rottman et al., 1997). Mutant gene variant CCR5-del32 does not produce any functional protein (Samson et al., 1996). Therefore, reduced number of the relevant receptors on the cell surface is expressed by the heterozygous CCR5 genotype. The CCR5-del32 homozygous individuals, having functionally inactive receptors, display no clinical symptoms and appear to be unaffected (Carrington, Dean, Martin, & O’Brien, 1999).

Mononuclear cells infiltration to pancreatic islets and the progressive Th1 cell-mediated destruction of insulin-producing islet β-cells herald the onset of autoimmune type I diabetes in NOD mice (Gill, Jaramillo, Ma, Laupland, & Delovitch, 1995). The NOD mouse is an animal model of type I diabetes mellitus, which shows many of the character-
istics of human type I diabetes (Shimada, Charlton, Taylor-Edwards, & Fathman, 1996). Temporal expression of certain CC-chemokines, particularly MIP-1α and its receptor, CCR5, in the pancreas of NOD mice contributes to the development of insulitis and spontaneous type I diabetes mellitus (Cameron et al., 2000). Recent population studies in humans have indicated that there is no association of the CCR5-del32 allele with type I diabetes mellitus (Szalai et al., 1999).

Type II diabetes mellitus has a multifactorial aetiology. Insulin resistance is common and most often caused by obesity, aging, and genetic factors. Genetic factors appear to be the major determinants for the development of type II diabetes, because genetically determined intracellular post-receptor defects likely play a role. It is concluded that dysfunction of the sympathetic nervous system could cause a predisposition to obesity and type II diabetes mellitus (Ahrén, 2000; Nonogaki, 2000).

In the present study, we have determined the CC-chemokine receptor 5 mutant allele Δ32 (CCR5-del32) frequency in Estonian types I and II diabetes mellitus patients, and tried to find how the CCR5 polymorphism affects the onset and course of diabetes.

2. Subjects and methods

2.1. Subjects

Previously, we have determined the frequency of the CCR5-del32 mutation in a control group, which consisted of 504 healthy unrelated native Estonians [age 14–94 (54.1 ± 4.1) years] (Kalev, Mikelsaar, Beckman, Tasa, & Pärlist, 2001). Criteria of native Estonians: all the four grandparents of the participants had to be ethnically pure Estonians. These four grandparents have been settled in the same region, as well as their ancestry from time immemorial. Contacting with individuals, completing a questionnaire, and collection of blood samples from each participant were done by using the aid of general practitioners.

Thirty-eight patients with type I diabetes mellitus [age 15–62 (35.1 ± 13.9) years] and 111 patients with type II diabetes mellitus [age 42–77 (63.0 ± 8.1) years] were investigated. The mean age at the onset of type I diabetes was 24 years (range 4–57 years) and the onset of type II diabetes was 54 years (range 21–77 years). Diabetes was diagnosed according to the World Health Organisation criteria, based on an oral glucose tolerance test. Retinopathy was diagnosed by fundoscopy, nephropathy in the presence of overt proteinuria, and neuropathy by physical examination. Type II diabetes patients were collected through the regular ambulatory examination at the Department of Internal Medicine of the Tartu University Clinics from December 1999 to January 2000, and type I diabetes patients in the study group were hospitalized due to undercompensation from September 1999 to May 2000 in the Tartu University Clinics, Department of Internal Medicine and in Magdalena Hospital in Tallinn, Estonia. The human studies were approved by the Committee of Ethics of Tartu University.

2.2. Molecular genetic determination of the CCR5-del32 mutation

Total genomic DNA was extracted from 5 ml of venous blood (EDTA added as an anticoagulant) by the phenol extraction procedure. The genotyping of CCR5 was performed by genomic DNA amplification by PCR and with subsequent analysis of the electrophoretic patterns of the PCR products (Samson et al., 1996).

The following primers are used: forward: 5′ CCTGGGCT-GTCGTCCATGCTG 3′, reverse: 5′CTCATCTAGA-GCCATGTGCAACAATCT 3′.

PCR conditions were: 30 cycles; annealing at 62 °C for 1 min; elongation at 72 °C for 1 min; denaturation at 93 °C for 1 min. To increase separation of the wild-type allele and allele carrying the deletion, the 736-bp PCR product was cleaved with EcoRI into a 332-bp constant fragment and 404- or 372-bp fragment for the wild-type or mutant alleles, respectively, and separated in 10% polyacrylamide gel. Bands were detected by ethidium bromide staining.

2.3. Statistical methods

Allele frequencies were calculated by allele counting. The fit to the Hardy-Weinberg equilibrium and differences between population samples were analysed using a χ²-test. The student t-test and Whitney–Mann U-test for unpaired samples were used to test differences in late complications and concomitant diseases. P < .05 was chosen as the level of significance. To test the influence of genetic factors on the number of late complications, the one-sided t-test was used. To control the influence of the disease duration on the

| Table 1 |
| CCR5-del32 genotypes and allele frequencies of controls and patients with types I and II diabetes mellitus |
| | Control subjects | Type I | Type II |
| | n | 504 | 38 | 111 |
| CCR5 +/Δ32 | 117 (23.2 %) | 11 (28.9 %) | 24 (21.6 %) |
| CCR5 Δ32/Δ32 | 16 (3.2 %) | 0 | 0 |
| Allelic frequency of Δ32 | 0.148 | 0.141b | 0.108c |

n: number of observations.
Control group consisted of unaffected and unrelated native Estonians (Kalev et al., 2001).

b P=.305; control subjects compared with type I diabetes mellitus patients.
c P=.197; control subjects compared with type II diabetes mellitus patients.
clinical course of the diabetes, the multiple linear model was used.

3. Results

The results of the allele frequencies are presented in Table 1. The frequency of CCR5-del32 mutation was not statistically different in types I and II diabetes and the control group of healthy unrelated native Estonian inhabitants. However, a significant association ($P < .05$) was found between CCR5 receptor gene polymorphism and the clinical course of type I diabetes, which starts earlier and lasts longer by the moment of our study in the case of wild-type CCR5 (CCR5 +/+) (Table 2). The average number of complications was 1.63 in group CCR5 +/+ (S.D. 1.08) and 0.91 in the mutant genotype group CCR5 +/D32 (S.D. 0.83); the difference of means was significant ($P < .03$).

In type II diabetes, the receptor gene polymorphism does not affect the age of onset and the clinical course of diabetes. However, a significant difference ($P = .0006$) existed in the frequency of concomitant diseases such as acute and chronic diseases, obesity and neoplasias, rheumatic and thyroid diseases, and others, which were more frequent in patients with a CCR5 wild-type genotype. The detailed list of complications that occurred in type II diabetes mellitus patients with CCR5 wild-type and mutant genotypes is shown in Table 3. There were no statistically significant differences between CCR5 wild-type and heterozygous genotypes.

4. Discussion

In this part of our work, we have found no differences in the frequency of CCR5-del32 between nondiabetic individual and diabetes mellitus patients. This confirms that the CCR5 gene in humans is not directly associated with the aetiology of either type I or type II diabetes.

However, we revealed the association between CCR5 polymorphism and the clinical course of type I diabetes. The mean onset of the disease was 5.4 years earlier in CCR5 wild-type homozygotes compared to CCR5-del32 heterozygotes, and has lasted for about 8.5 years longer by the starting moment of our study. The mean age in the different genotype groups at the study moment was not statistically different. According to the multiple linear regression model, the influence of disease duration was statistically proven ($P < .05$), but the influence of genetic markers was not statistically significant ($P > .05$). However, starting from a standard error resulting from using our data...
in the model, we can calculate a factor, which indicates that, if the number of patients could be six times more, the same regression coefficient could be statistically significant. Then, the influence of genetic factors could be proven. Hence, it can be regarded as a tendency (because of the small group of patients) that, in the case of type I diabetes, the frequency of late complications is increased in CCR5 wild-type (+/+ patients).

It might not be just a coincidence that the expression of CCR5 in endothelial, smooth muscle, and microglial cells matches the target tissues of complications of type I diabetes. From these data, we can draw a conclusion that the reduced CCR5 concentration on the cells in the case of CCR5 heterozygous genotype may be protective in hyperglycemic conditions against disease complications. We can not explain the whole process of late complications’ formation, but we want to add a small piece to the concept. It has been determined that the chemokine binding selectivity of CCR5 is mediated by the second extracellular loop, while affinity-sensitive binding is dependent on the N-terminus and the first extracellular loop (Murphy, 1996; Ward, Bacon, & Westwick, 1998). In hyperglycemic conditions, an unspecific glycosylation could make the ligand-receptor complex more stable and extend the time of the G protein’s activation. Hypothetically, it might be that the biological signal molecules, maybe steroid hormones (for example, progesterone), pass through the cell membrane in association with CCR5 affinity-sensitive part, and inhibit the phospholipase C cascade (Vassiliadou, Tucker, & Anderson, 1999). Hence, the Ca^{2+} level-enhancing mechanisms in the cell are disturbed. This does not allow the triggering of a phosphorylation and subsequent cellular responses. When the Ca^{2+} level in the cell does not increase, the phospholipase A_2 (PLA_2) activation does not take place (Nelson & Cox, 2000). If the PLA_2 normal functions (cell membrane phospholipids decomposition, biosynthesis, and remodelling) are suppressed, the cytoskeletal reorganizations take place. This leads to higher physical lability of membrane structures prior functional changes in cells. Maybe concurrently the integrin genes are switched on and the rapid integrin-mediated adhesion is induced (Luster, 1998). It can be supposed that diabetic late complications, founded on changes in membrane structures of endothelial, smooth muscle and microglial cells, arise by the CCR5 involvement. Under long-lasting hyperglycemic conditions, these cell membrane changes may be amplified by derangement in antioxidants/oxidants balance, and overcome physiological limits followed by substantially increased free radical formation and lipid peroxidation (Bautista, 2001). This may also lead to the translocation and activation of redox-dependent nuclear transcription factors (Lakshminarayan, Drab-Weiss, & Roebuck, 1998). In addition, for chemotaxis, extracellular calcium is required to ensure proper reversible interaction of adhesion molecules with the substratum (Mandeville & Maxfield, 1997).

Thus, we can speculate that the CCR5 gene may be a modifying gene for the clinical course of human type I diabetes. This hypothesis might be a subject for further investigation, when data from larger patient groups become available.

In type II diabetes pathophysiology, there is no evidence for the participation of the immune-reactive cells (CD4+, CD8+ T cells, and monocytes/macrophages). The Δ32 polymorphism of the CCR5 gene was not associated with the frequency of type II diabetes in this study. However, in type II diabetes in the CCR5 wild-type genotype, the frequency of concomitant diseases (obesity, thyroid and autoimmune diseases, neurologic disorders, neoplasias, and other concomitant diseases) per patient is increased compared to CCR5 heterozygotes (P = .0006). We propose that it could be taken as an evidence that CCR5 polymorphism in type II diabetes is somehow related to the immunologic homeostasis of individuals.

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