ASSOCIATION OF INTERCELLULAR ADHESION MOLECULE-1 469 E/K POLYMORPHISM WITH TYPE 1 DIABETES

Cristian Guja[1], *Marilyn Merriman*[2], *F. Cucca*[3], *S. Marshall*[4], *K. Welsh*[4], *J.A. Todd*[2] *and Constantin Ionescu-Tîrgovişte*[1]

1 – Clinic of Diabetes, Institute of Diabetes, Nutrition and Metabolic Diseases
“N. Paulescu”, Bucharest, Romania.
2 – JDRF/Wellcome Trust Diabetes and Inflammation Laboratory,
Cambridge Institute for Medical Research, Cambridge, UK.
3 – Dipartimento di Scienze Biomediche e Biotecnologie, University of Cagliari, Italy
4 – Tissue Typing Laboratory, Oxford Transplant Centre, Oxford, UK.

Abstract

Type 1 diabetes is a disease with autoimmune pathogenesis, mainly conditioned by genetic susceptibility factors. Recent studies (mainly on NOD mice) showed the importance of Intercellular Adhesion Molecule-1 (ICAM-1) in the autoimmune destruction of pancreatic □ cells. We checked the possible association of ICAM-1 with type 1 diabetes genetic susceptibility on 204 Romanian diabetic simplex families, country with one of the lowest incidences of type 1 diabetes in Europe. For this we used the Sequence Specific Primer-Polymerase Chain Reaction (PCR-SSP) technique to type 469 E/K and 2412 G/R alleles of ICAM-1 gene on chromosome 19p13.3-2. The data were analysed using the Transmission Disequilibrium Test (TDT).

We found for the Romanian population a significant increased transmission of 469 E allele to diabetics (63.54%, p=0.00011). We tried to replicate the data on 243 Sardinian and 424 UK families. We could confirm the association of 469 E allele with type 1 diabetes susceptibility for the Sardinian families (p=0.013) but not for the UK families (p=0.87). However, for these populations we found a trend of increased transmission of 469 E allele from mothers to diabetic offspring. The combined analysis of x families showed a significant increased maternal transmission (59.42%, p=0.0007). We did not observe differences in transmission for offspring stratified according to age at onset.

In conclusion we found an association of ICAM-1 469E allele with type 1 diabetes risk for the Latin Romanian and Sardinian populations but not for the UK population.

Introduction

Type 1 diabetes mellitus (T1DM) is a common chronic disease characterised by the autoimmune destruction of insulin secreting pancreatic β cells. The destruction is complete and results in absolute insulin dependency. T1DM is a multifactorial disease the aetiology of which involves both genetic and environmental factors (1,2,3).

Genetically, T1DM is a complex, polygenic disease, with multiple susceptibility and protective alleles interacting with each other. The study of candidate genes identified the major two susceptibility genes for T1DM: *IDDM1* encoded in the HLA region of the Major Histocompatibility Complex (MHC) on chromosome 6p21 (1,4,5) and mapped to the *DRB1, DQB1* and *DQA1* loci (6,7) and *IDDM2* encoded by the insulin gene region mapped to the *VNTR* region 5' of the insulin gene on chromosome 11p15 (8,9). This approach also ravelled the association of other three loci, all with smaller contributions to T1DM susceptibility: *IDDM12* – the CTLA4 (cytotoxic T lymphocite associated protein 4) gene (10,11); the PTPN22 (lymphoid tyrosine phosphatase) gene (12,13) and the IL2RA/CD25 gene (14). Using linkage analysis strategies by whole genome study, some other regions of the human genome were linked with T1DM (15,16,17,18,19) but none of these putative genes has been identified yet.

We previously reported the *IDDM1* and *IDDM2* status for the Romanian population, one with one of the lowest T1DM incidences in Europe (20,21). In this study we tested for association with T1DM for the Romanian population the *Intercellular Adhesion Molecule-1* (*ICAM-1*) gene on chromosome 19p13.3. For this, we typed 204 Romanian type 1 diabetic families using a unified method of genotyping multiple HLA and non-HLA polymorphisms under identical conditions employing the Sequence Specific Primer - SSP-PCR technique (22,23). Subsequently, we tried to replicate the significant results (an association of *ICAM-1* 469 E/K polymorphism) on 243 Sardinian simplex and multiplex and 424 UK multiplex T1DM families.

Materials and Methods

Families selection 204 Romanian T1DM families were genotyped in this study. We chose the Romanian population because of its the very low reported incidence of T1DM (20,24). Informed consent was obtained prior to blood collection for DNA extraction from all subjects involved and the study was approved by the local ethics committee. The ascertainment of families was as previously described (21). Most were simplex families (196) but there were 3 multiplex families and 5 families with one parent also affected. Most were 4 member families (144) and the other (60) were 3 member families. Overall there were 756 individuals; 371 M (49.07%)/385 F (50.93%). From these families the patient cohort comprised 212 type 1 diabetic patients (106 M/106 F) with the onset of disease between 9 months and 43 years. The median age at the onset of disease was 12.1±6.7 years. Most patients (166 - 78.3%) had diabetes onset under 17 years age. There were 544 unaffected individuals (parents and siblings), 265 M (48.71%)/279 F (51.29%). The 257 Sardinian families were all single case families, except for 13 who had a parent affected, and 10 who had a second sibling affected (25). The ascertainment of 424 UK families was previously described (17).

Selection of genes We selected for analysis the *ICAM-1* gene due to its important functions in the immune response and due to its location on a chromosomal region previously reported to be associated with

T1DM (17). There are several polymorphisms of ICAM-1 gene (chromosome 19p13.3-2) in humans (26). We typed two of these polymorphisms: the 657A/G substitution in exon 4 (241G/R polymorphism in the amino-acid sequence) and the 1548A/G substitution in exon 6 (469E/K polymorphism in the amino-acid chain). The two polymorphisms we typed were previously described (23,27).

Primer design The primers used for ICAM-1 typing of the Romanian families were as previously reported (27). For ICAM-1 typing of Sardinian and UK families we slightly redesigned the primers in order to facilitate gel interpretation. All listed primers are 5' to 3'. The sequences of the primers used for typing of the 469 E/K polymorphism were (Genebank accession number X59288): E allele forward primer TGGACGAGAGGGATTGTCC (annealing position 1336-1354); reverse primer GCACATTCACGGTCACCTC (annealing position 1566-1548), 50 ng/PCR amplification, 231 bp amplicon; K allele forward primer TGGACGAGAGGGATTGTCC (annealing position 1336-1354); reverse primer GCACATTCACGGTCACCTT (annealing position 1556-1548), 50 ng/PCR amplification, 231 bp amplicon. For both alleles we used an internal control for validation of amplification: *HLA-DRB1* intron 3 amplicon of 796 bp, forward primer TGCCAAGTGGAGCACCCAA with annealing position exon 3 amino acid 173-179; reverse primer GCATCTTGCTCTGTGCAGAT with annealing position in exon 4 amino acid 193-200, both 12 ng/PCR amplification.

DNA extraction DNA extraction for the Romanian, Sardinian and UK families was done as previously described (21).

PCR amplification and electrophoresis All *ICAM-1* genotyping was done as follows: Genomic DNA was amplified in 13 μl reactions as previously described (22,23,28). Subsequently, PCR products were electrophoresed through 1% agarose gels containing 0.5 μg/ml ethidium bromide in 0.5xTBE buffer (89 mmol Tris base, 89 mmol boric acid, 2 mmol EDTA, pH 8.0) for 20 - 30 min at 15 V/cm.

Following electrophoresis, the products were visualised with UV illumination and the gel photographed with a Polaroid camera. Gel interpretation was simple, requiring the scoring of the presence or absence of an amplicon. For validation of PCR amplification, all reactions included an internal control, a 796 or 256 bp product depending on the HLA amplicon size, as previously detailed (22,23,28).

Statistical analysis Data was initially analysed using the Transmission Disequilibrium Test (TDT) (29). For all the new polymorphisms that were tested on the Romanian families, the p value was corrected according to the Bonferroni correction method and is marked as p* in all the tables. All p values < 0.05 were considered significant. Analysis of the transmission of ICAM-1 469 alleles to affected and unaffected individuals from Sardinian, UK, US and NI families was done also by TDT testing.

Results

We initially analysed on the Romanian dataset of families 37 polymorphisms (most of them SNP's) in 17 immunomodulatory genes. Since these polymorphisms were tested for the first time on Romanian T1DM families, we corrected all the p values of the TDT analysis using the Bonferroni method (thus multiplying the obtained p value with 74 x 2 - the sum of the number of loci multiplied by the number of alleles at each locus, all multiplied by 2 since we studied both maternal and paternal transmissions as well as transmissions from both parents). The only significant result was an association of the 469 E allele of *ICAM-1* (30).

Our data (Table 1) show a significantly increased transmission of *ICAM-1* 469 E allele to the diabetic probands (63.54%, corrected p=0.01628) compared with their unaffected siblings (52.71%, p=0.3). The diabetic siblings inherit this "susceptibility" allele especially from their mothers (69.81% maternal transmission compared with just 45.95% paternal transmission). We also found an increased transmission of *ICAM-1* 241 R allele to the affected (62.71%, p=0.034, corrected p >1) compared with the unaffected (53.49%, p=0.38), though not significant after the Bonferroni correction. The transmission of *ICAM-1* 241/469 haplotypes in the affected show a protective haplotype 241G/469K, (32.39% transmission, p-2.75e-05) and a susceptibility haplotype 241G/469E, (63.57% transmission, p-0.0013). The transmission of these haplotypes was 47.77% and 51.16% respectively to the unaffected.

We further studied the transmission of ICAM-1 469 E allele to affected offspring stratified by their DR types. We found a significantly increased transmission of this allele just in the DR4/4 and 4/X diabetic probands (75.47%, corrected p<0.01) For the DR3/X and DR3/4 diabetic patients, the transmission was increased but not significantly after correction (60.97% transmission, p*>1 for DR 3/X and 54.05% transmission, p*>1 for DR ¾). These data show that, for the Romanian population, the 469 E "susceptibility" allele is transmitted preferentially to HLA-DR4 diabetic offspring from heterozygous parents, suggesting that there may be an interaction of these two loci for this population.

To confirm our findings, we tried to replicate our results on four other populations. For this we typed for ICAM-1 241 G/R and 469 E/K polymorphisms 243 Sardinian simplex and multiplex (941 individuals, 264 affected) and 424 UK multiplex (1888 individuals, 904 affected) families. On the Sardinian families we could confirm that there is a significantly increased transmission of 469 E allele to affected (57.14%, p=0.013) compared with their unaffected siblings (53.37%, p=0.19), though it is not as strong as for the Romanian population (Table 3). We found the same pattern of increased transmission of 469 E allele preferentially from mothers (65.52%, p=0.012) compared with the paternal transmission (57.75%, p=0.12). We could identify the same protective 241G/469K (41.11% transmission, p=0.017) and susceptibility 241G/469E (59.21% transmission, p=0.013) haplotypes. The transmission of these haplotypes was 46.89% and 53.42% respectively in the unaffected siblings. We could not confirm, for the Sardinians, the preferentially transmission of 469 E susceptibility allele to DR4 positive diabetic probands (51.51% transmission, p=0.5) but we found a preferentially transmission to DR3 positive diabetics (64.34%, p=0.00071). We could not confirm the increased transmission of 469 E allele to diabetics on 424 UK multiplex families (47.99% transmission, p=0.87 compared with 52.73% transmission to unaffected siblings, p=0.27) (Table 2).

The combined analysis (Table 2) showed no effect of the *ICAM-1* 469E allele on T1DM susceptibility on this dataset of 885 T1DM families, but we noticed a trend of increased transmission of this allele to diabetic siblings from their mothers (59.42% transmission, p=0.0007.

Discussion

In this study we used a candidate gene approach to ascertain the genetic component of T1D susceptibility on the Romanian population, using 204 Romanian Type 1 diabetic families. For this we used a SSP-PCR technique which enabled us to test a large number of HLA and non-HLA polymorphisms under identical PCR amplification conditions. Previously we tested the Romanian families for *IDDM1* and *IDDM2* in order to validate genetically for Type 1 diabetes this population. The results confirmed the major role of *HLA-DR/HLA-DQ* alleles and of INS-*VNTR* alleles in T1DM susceptibility for the Romanian population (21).

From all the new candidate genes analysed on the Romanian population we found a significant association with just one, an *ICAM-1* polymorphism. Intercellular Adhesion Molecule11 (ICAM-1 or CD 54) is an integral membrane protein of the immunoglobulin super-family. It is a heavily glycosylated (90 000 kd), single chain cell surface molecule with five extracellular immunoglobulin like domains (3-7 nm per Ig domain) (31,32,33). ICAM-1 is expressed normally on B and T lymphocytes, epithelial cells, endothelial cells and fibroblasts (a distribution similar to that of MHC class II antigens). Cytokines and bacterial products, released at inflammatory sites, induce ICAM-1 mRNA and protein expression in a wide variety of cell types (31,34,35), including pancreatic β cells (36). ICAM-1 ligands are the β_2 Integrins (members of the integrin family of cell surface receptors): LFA-1 (Leukocyte Function Associated Antigen 1 ⇔ CD11aCD18) and Mac-1 (CD11bCD18) which are expressed on most leukocytes (33,34). ICAM-1/LFA-1 interaction leads to enhancement of T helper cell activation (31,34,37), leukocyte adhesion to fibroblasts, endothelial and epithelial cells with extravasation (31,34), natural killing, antibody dependent and T cell mediated cytotoxicity (34). The contact of T cell receptors with cells bearing specific antigen generates intracellular signals that lead to the conversion of LFA-1 to a high avidity state and regulates LFA-1/ICAM-1 dependent adhesion in an antigen specific manner (34). All these show that ICAM-1 and its ligand (LFA-1) have essential functions in the immune response. There are several reports of ICAM-1/LFA-1 involvement in different autoimmune or with immune pathogeny diseases (38,39,40).

A circulating form of ICAM-1 has been detected in human serum that appears to result from the proteolytic cleavage of membrane ICAM-1 (a truncated form of ICAM-1, lacking the transmembrane and cytoplasmatic domains). Soluble ICAM-1 inhibits LFA-1/ICAM-1 interaction and antagonises ICAM-1/LFA-1 mediated cellular events (41,42,43) thus acting as an anti LFA-1 antibody. Monoclonal antibodies (mAb) anti ICAM-1 and anti LFA-1 inhibit granulocyte migration, antigen induced lymphocyte proliferation and T-cell mediated cytolysis in vitro (34,42). There are reports of using anti ICAM-1 monoclonal antibodies to prevent renal allograftgraft rejection (44) or in refractory rheumatoid arthritis (45).

Several studies on animal models reported an involvement of ICAM-1 in the pathogenesis of Type I diabetes. The expression of ICAM-1 on pancreatic β cells accelerates their destruction by cytotoxic T-cells in NOD mice (36). Treatment of NOD mice with monoclonal antibodies against ICAM-1 led to a permanent inhibition of the development of diabetes (46). Exogenous soluble ICAM-1 (sICAM-1) has a potent inhibitory effect on the development of autoimmune diabetes in NOD mice (47).

ICAM-1 is therefore a natural candidate gene for T1DM. The *ICAM-1* gene is located on chromosome 19p13.3-2 and was completely sequenced (32,33,48). Three single nucleotide polymorphisms (substitutions) have been identified: in exons 2, 4 and 6, leading to aminoacid changes in the first, third and fifth extracellular immunoglobulin like domains (26,49). These aminoacid changes - which could lead to conformational changes of ICAM-1 molecule - are supposed to have functional effects since the first and second Ig like domains are LFA-1 binding site, the third Ig like domain is Mac-1 binding site and changes in the fourth and fifth Ig like domains decrease LFA-1 binding several-fold (26,35). Previous studies showed that the exon 2 polymorphism is absent in Caucasians (23). Therefore we typed *ICAM-1* only for the exon 4 and 6 polymorphisms. The exon 4 polymorphism is an 657 A/G substitution which leads to a G/R polymorphism in position 241 of the ICAM-1 molecule. The exon 6 polymorphism is an 1548 A/G substitution and generates an E/K polymorphism in position 469 of ICAM-1 molecule. Associations of 241 and 469 polymorphisms of *ICAM-1* with renal graft rejection (27), Crohn's disease and ulcerative colitis (50) were previously reported.

In our study we found a significantly increased transmission of *ICAM-1* 469 E allele to diabetics in 204 Romanian diabetic families and we could replicate the results on 243 Sardinian diabetic families. For both populations, the diabetic siblings inherited the susceptibility allele more frequently from their mothers. The study of 424 UK multiplex families failed to reconfirm the increased transmission of 469 E allele to diabetic siblings from their heterozygous parents. In the present study, the combined analysis of the five populations studied, leads to a 52.28% transmission of 469E allele to affected (p=0.1). Looking just at the maternal transmissions, we found overall a 59.42% transmission of 469 E allele from heterozygous mothers to affected offspring (p=0.0007). Since we tested the transmissions to affected as well as to unaffected offspring, we could exclude a segregation distortion effect. In our study we could not confirm the findings of a Japanese study (51) which suggested a possible association of ICAM-1 469 E/K polymorphism just for the late onset diabetic patients (mean age 46.8 years) but not with the young onset patients (mean age 11.6 years).

Similarly designed studies found no association of ICAM-1 469 E/K polymorphism with T1DM susceptibility for the Danish (52) and Finnish populations (53,54). However, the combined analysis of the association data for the Romanian, Danish and Finnish T1DM families (55) suggested an association with type 1 diabetes (overall transmission to diabetics 55% with p_{TDT}=0.013), the result being explained only by the strong effect observed on the Romanian families. It is interesting to note that, more recently, a weak association of the 469E allele with T1DM was reported on a case/control dataset from Poland (56), the authors indicating also a possible protective effect of the 241R allele, this effect being however in the opposite way than that observed on the Romanian families. In an effort to finally elucidate the role of *ICAM-1* in T1DM genetic susceptibility, Nejentsev et al. studied the G241R and E469K SNPs on a dataset of 3695 families from Finland, UK, USA, Norway and Romania. The results infirmed the association of 469E allele with T1DM but confirmed the possible protective effect of 241R (p_{TDT} = 0.03) (57).

The results of our study, correlated with the known function of ICAM-1 in human immune responses and its involvement in the pathogenesis of murine insulin dependent diabetes, strongly suggest that ICAM-1 could be involved in the pathogenesis of T1DM in humans and that certain polymorphisms of ICAM-1 gene could be aetiological. The fact that we could confirm our initial findings in the Romanian population on a genetically close population (Sardinian) but not on the UK population suggests either that ICAM-1 469 polymorphism have just a small contribution to T1DM susceptibility and just for some populations, or that the real susceptibility mutation is somewhere else in ICAM-1 gene region on chromosome 19p13.

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