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Genetic variation in ICOS regulates mRNA levels of ICOS and splicing isoforms of CTLA4

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Abstract

Genetic and functional studies suggest that polymorphism in cytotoxic T lymphocyte-associated antigen-4 (CTLA4) and inducible costimulator (ICOS) genes, both reported to harbour autoimmune susceptibility loci, could regulate the immune activation through affecting their expression and splicing of CTLA4. To address this, we studied expression of CTLA4 and ICOS and the role of polymorphisms in the gene region by measuring the relative amounts of transcripts, including the soluble CTLA4 (sCTLA4) splicing isoform in healthy volunteers. We combined a physiologically relevant *in vitro* activation for human $CD4^+$ T lymphocytes and a quantitative RT-PCR. The susceptibility allele CT60G in CTLA4 gene was confirmed to be associated with a decreased amount of sCTLA4, but only in resting cells. During the T cell activation two genetic variants in ICOS gene, IVS1+173T/C and c.1624C/T, affected expression of CTLA4 isoforms and ICOS, respectively. We could not confirm that the level of sCTLA4 is down-regulated following T lymphocyte activation, instead the levels of CTLA4 splicing isoforms correlated to each others. Our results indicate that genetic variation in this gene region regulates the expression of both CTLA4 and ICOS and not only the splicing of sCTLA4 as suggested earlier.

Keywords: ICOS; CTLA4; Regulatory SNP; Functional polymorphism; T lymphocytes; Autoimmune susceptibility

1. Introduction

Chromosomal region 2q33 harbouring the human CD28 cosignal receptor gene family, CD28, cytotoxic T lymphocyte-associated antigen-4 (CTLA4) and inducible costimulator (ICOS), has been suggested to carry a predisposing gene locus for several autoimmune diseases (Gough et al., 2005), such as type 1 diabetes, Graves' disease and coeliac disease. The molecules have a crucial role during the T lymphocyte activation and its regulation: CTLA4 inhibits and ICOS stimulates the activation. Hence they are good functional candidates for autoimmunity and transplantation tolerance studies. Evidence based on linkage and genetic association studies has so far pointed to CT60 polymorphism near CTLA4 as the most promising susceptibility locus for type 1 diabetes and Graves' disease (Ueda et al., 2003). However, we and others have shown that in coeliac disease (Haimila et al., 2004) and in multiple sclerosis (Bonetti et al., 2004) polymorphisms near ICOS might also be likely candidates. All single nucleotide polymorphisms (SNP) in this gene region that are sufficiently common for candidates are located outside of the sequences coding for amino acids on the final protein product. This means that the functional susceptibility polymorphism is most likely a regulatory SNP affecting the amount of the product. Because of the complex genetics behind autoimmune diseases and the strong linkage disequilibrium in this chromosomal region, it may not be easy to pinpoint the actual predisposing polymorphism(s). However, studies looking for functional effects of the polymorphisms could reveal some relevant candidates.

First functional studies of this locus focused on CTLA4 and its non-synonymous exon 1+49A/G and promoter −318C/T SNP which appeared to have an influence on the amount of CTLA4 protein (Kouki et al., 2000; Ligers et al., 2001; Maurer et al., 2002; Wang et al., 2002; Anjos et al., 2002). More recently, it was demonstrated that a candidate susceptibility polymorphism might change the ratio of CTLA4 splicing isoforms (Ueda et al.,

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2003; Atabani et al., 2005). However, conflicting results have also been reported (Anjos et al., 2005).

The splicing isoform of CTLA4 from which the third exon coding for the transmembrane domain is missing (Magistrelli et al., 1999) is a soluble protein named sCTLA4. It binds the same B7 family ligands as the full-length isoform (flCTLA4) and may inhibit immune activation as demonstrated in mixed lymphocyte reaction (Oaks et al., 2000). Also, elevated levels of sCTLA4 are detected in autoimmune disorders (Pawlak et al., 2005). This suggests that sCTLA4 might be a regulator or an active component of immune response. There is also evidence that the levels of sCTLA4 decrease during immune activation (Magistrelli et al., 1999; Oaks et al., 2000), but not so much in systemic lupus erythematosus patients (Wong et al., 2005). Hence, it is possible to create a hypothesis that sCTLA4 regulates T cell immune activation and that its levels are regulated by genetic variation in the CD28 gene complex. This could provide us with a mechanistic explanation for the associated gene polymorphisms. We here addressed this question by studying CTLA4 and ICOS expression levels in human peripheral $CD4^+$ T lymphocytes obtained from individuals with different genetic variants in the CTLA4 and ICOS genes. We focused on mRNA levels, as we can assume that differences in mRNA immediately after immune activation should reflect more directly genetic effects than measurements of protein levels or cellular activation, which are subject to more complex regulation.

2. Materials and methods

2.1. Samples and stimulation of $CD4^+$ T cells

$CD4^+$ T cells were separated from fresh whole blood samples using the RosetteSep™ Human $CD4^+$ T Cell Enrichment Cocktail (StemCell Technologies Inc., Vancouver, BC, Canada) immediately after drawing the blood. Enrichment is based on negative selection leaving the desired cells untouched. Separation was done according to the manufacturer's instructions except the density gradient centrifugation which was done at $900 \times g$. Routinely >95% purity was achieved as checked by flow cytometry ($CD4^+CD14^-$).

Activation of cells was done with anti-CD3 antibody (UCHT1) and CD80-muIg fusion protein (both from Ancell Corporation, Bayport, MN, USA). These biotin-conjugated proteins were attached on avidin (Pierce Biotechnology Inc., Rockford, IL, USA) coated 24-well plates (activation method is described more detailed elsewhere, Autero manuscript in preparation). Mouse IgG with irrelevant specificity was used as a negative control (Biotin Mouse IgG2a Isotype Control; eBioscience, San Diego, CA, USA). 2.5×10^6 $CD4^+$ T cells were added per well in complete RPMI-1640 (10% fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin) and stimulated for different time periods from 1 to 24 h. The stimulations were done in triplicates whenever the cell number per sample was large enough (79% of all stimulations).

All study subjects were healthy adults who voluntarily participated in this study which was approved by the ethical committee of the Hospital District of Helsinki and Uusimaa (HUS).

2.2. Flow cytometry

Basic protocols were used for surface staining of $CD4^+$ T cells. The following FITC- or PE-labelled monoclonal antibodies were used: CD4 from eBioscience (San Diego, CA, USA), CD14 from BD PharMingen (San Diego, CA, USA), CD25 from Miltenyi Biotec (Bergisch Gladbach, Germany) and CD69 from ImmunoTools (Friesoythe, Germany). Appropriate isotype controls were included in all experiments. Flow cytometry analysis was performed on Becton Dickinson FACSCalibur™ and data were analyzed using the CellQuest™ software (BD Biosciences, San Jose, USA).

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted from frozen cell samples with RNeasy Mini Kit and DNase treatment was done with RNase-Free DNase Set (both from Qiagen, Hilden, Germany). Reverse transcription (RT) was performed with High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) according to the instructions provided by the manufacturer. RNA concentration in each RT reaction was 5 ng/μl and RNase inhibitor (Applied Biosystems) was added at 1 U/μl. Reactions were primed by random primers and synthesis was done by MultiScribe RT enzyme both provided with the cDNA kit.

To control the absence of genomic DNA-RT reactions were performed on a few samples without MultiScribe RT enzyme. No amplification could be detected in these reactions by quantitative PCR.

2.4. Quantitative real-time PCR

To quantitate relative amounts of mRNA molecules ABI PRISM 7000 Sequence Detection System and TaqMan® chemistry were employed (Applied Biosystems, Foster City, CA, USA). PCR reaction components and conditions were as recommended by Applied Biosystems. TaqMan® Gene Expression Assay was used for ICOS detection (Hs00359999_m1). The primers and probes for flCTLA4 and sCTLA4 (Ueda et al., 2003) were purchased from Sigma. 18S ribosomal RNA was used as an endogenous control gene (TaqMan Ribosomal RNA Control Reagents from Applied Biosystems) and it was multiplexed with the actual targets. All amplifications were done in triplicates. The same standard curve, diluted from total RNA from peripheral blood mononuclear cells, and non-template controls were included in every run. To calculate the amount of target mRNA in a sample the relative standard curve method (Applied Biosystems) was used. Briefly, input amounts were calculated using standard curves and relative amounts (from now on referred as the actual RNA level) were obtained by normalizing to the endogenous control. These results were proportioned to standards and thereby depicted as manifolds of them. Also activation-induced changes in the expressions were calculated: actual RNA level at time point of interest/actual level at 0 h = fold change from 0 h.