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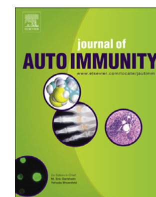

Short communication

Chromogranin A is a T cell antigen in human type 1 diabetes

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ABSTRACT

Chromogranin A (ChgA) is a beta cell secretory granule protein and a peptide of ChgA, WE14, was recently identified as a ligand for diabetogenic CD4 T cell clones derived from the NOD mouse. In this study we compared responses of human CD4 T cells from recent onset type 1 diabetic (T1D) and control subjects to WE14 and to an enzymatically modified version of this peptide. T cell responders to antigens were detected in PBMCs from study subjects by an indirect CD4 ELISPOT assay for IFN-γ. T1D patients ($n = 27$) were recent onset patients within one year of diagnosis, typed for HLA-DQ8. Controls ($n = 31$) were either 1st degree relatives with no antibodies or from the HLA-matched general population cohort of DAISY/TEDDY. A second cohort of patients ($n = 11$) and control subjects ($n = 11$) was tested at lower peptide concentrations. We found that WE14 is recognized by T cells from diabetic subjects vs. controls in a dose dependent manner. Treatment of WE14 with transglutaminase increased reactivity to the peptide in some patients. This work suggests that ChgA is an important target antigen in human T1D subjects and that post-translational modification may play a role in its reactivity and relationship to disease.

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1. Introduction

Autoreactive T cells directed toward β-cell antigens play a central role in the destruction of β-cells in human type 1 diabetes (T1D) and in the NOD mouse model of T1D. The presence of autoreactive T cells in individuals may indicate an ongoing attack on β-cells and can serve as an important biomarker for pre-diabetic status. This time-window in disease development, before overt hyperglycemia, is of key interest for therapeutic intervention strategies, such as antigen-specific tolerance induction, to prevent disease onset [1,2].

Using a proteomic strategy, we identified Chromogranin A (ChgA) as the target antigen for diabetogenic CD4 T cell clones, including the widely used BDC-2.5 T cell, derived from NOD mice [3]. The peptide WE14, a naturally occurring cleavage product of ChgA, is a weak antigen for these T cell clones *in vitro*, but the antigenic activity of the peptide is dramatically increased upon treatment with the enzyme tissue transglutaminase (TGase), which is known to covalently crosslink proteins through the formation of isopeptide bonds as well as catalyze glutamine deamidation reactions, such as those that occur in the posttranslational modification of gliadin in celiac disease. In the case of ChgA-reactive T cell clones from non-obese diabetic (NOD) mice, crosslinking rather than deamidation is involved in rendering WE14 more antigenic upon TGase treatment [4]. Since the amino acid sequences of human and mouse WE14 are identical, with the exception of one amino acid [5], we tested T cells from newly diagnosed T1D patients and controls for responses to human WE14 or a TGase-converted form of this peptide. We employed an indirect ELISPOT assay to compare responses to WE14 and TGase-modified WE14 in CD4 T cells from diabetic and control subjects.

2. Materials and methods

2.1. Study participants

Participants of both genders, aged 9–44 years, and typed for HLA DQ8, were recruited from patients, relatives, and volunteers attending the Barbara Davis Center for Diabetes, in accordance with protocols approved by the Colorado Multiple Institutional Review

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Board. Subjects with clinical disease of over one year duration at the time of blood draw were excluded from the study, as were control subjects who tested positive for one or more diabetes autoantibodies (insulin autoantibody [IAA], autoantibody to GAD65 [GADA], ICA512, or ZnT8A). An initial cohort (Cohort 1, see Table 1) was used to look at the effect of 20 and 40 μM doses of WE14 and TGase-treated WE14. A second cohort (Cohort 2, see Table 1) was recruited to examine a range of lower peptide concentrations.

2.2. Peptides

Peptides P1 (Proinsulin C19-A3; GSLQPLALEGSLQKRGIV), Insulin B9-23 (SHLVEALYLVCGERG), DR3-restricted GAD3 (GAD65 335-352; TAGTTVYGAFDPLLAVAD), DR4-restricted GAD4 (GAD65 554-575; VNFFRMVISNPAATHQDIDFLI), and R2 (IA-2 853-872; SFY LKNVQTQETRTLTQFHF) were synthesized at >95% purity (U. of Colorado Cancer Center Proteomics Core). A DQ8-restricted epitope of HA (Influenza Hemagglutinin MP 185-205; EEVDMTPA-DALDDFD), the kind gift of W. Kwok, Benaroya Inst., was synthesized at >95% purity (GenScript USA Inc. 860 Centennial Ave., Piscataway, NJ 08854,USA). WE14 (ChgA342-355; WSKMDQLA-KELTAE) was synthesized at >95% purity (Chi Scientific). All peptides were added to assays from stocks of 10 mM in DMSO. Pediacil® and Tetanus Toxoid were used as control antigens.

2.3. Transglutaminase treatment

Cohort 1: A solution of 250 μl of WE14 in PBS (5 mg/ml) was added to 4.75 ml of a reaction mixture containing 1.1 mM EDTA, 2.1 mM DTT, 52.6 mM NaCl, 52.6 mM HEPES (pH 8.0), 42.1 mM $CaCl_2$ and 105 mU/ml TGase (Sigma). This mixture was incubated for 4 h at 37 °C and then centrifuged at 1000 g for 10 min; the pellet was resuspended in 375 μl PBS and 10 μl aliquots were frozen at −80 °C. Cohort 2: A solution of 100 μl of WE14 in H_2O (5 mg/ml) was added to 400 μl of a reaction mixture containing 6.25 mM DTT, 62.5 mM NaCl, 62.5 mM Tris (pH 8.2), 12.5 mM $CaCl_2$ and 250 mU/ml TGase (Sigma). The solution was incubated for 7 h at 37 °C followed by freezing of 22 μl aliquots at −80 °C. Control samples (WE14 only, TGase only) were prepared in the same manner.

2.4. ELISPOT analysis

Indirect ELISPOT analyses were conducted as described previously [6], using the human IFN-γ ELISPOT kit (U-CyTech Biosciences, Utrecht, The Netherlands). Briefly, PBMCs from subjects were incubated with peptide or antigens for 48 h and subsequently transferred to plates coated with the anti-IFN-γ capture antibody followed by overnight incubation. Cells were removed and wells were washed. Spots were then formed by sequential incubations with the biotinylated second site anti−IFN-γ, gold-labeled goat anti-biotin, and a precipitating silver substrate; spots were enumerated with a Bioreader 4000 Pro X (BIOSYS, Karben, Germany). For each antigen tested, the total spots from three 96 well plates are added, reflecting spot forming cells per 9×10^5 total cells. In this study, a stimulation index (SI) of >2.0 was considered a positive response (SI = total spots for analyte/total spots for DMSO). All subjects were tested with ChgA peptide (WE14) and control peptides (tetanus, Pediacel). Due to cell number limitations, some subjects were not tested with all previously identified diabetes-related peptides although for each analyte the percentage tested was >66%.

Table 1
Demographics of subjects.

	Cohort 1		Cohort 2	
	Controls[a]	T1D[b]	Controls[a]	T1D[b]
Number of subjects	31	27	11	11
Age at blood draw (median, range)	25, 8−44	18, 9−39	26, 16−33	15, 10−23
Gender M/F	10/21	16/11	4/7	6/5
Antibody Positive (IAA, GAD65, IA-2, ZNT8)	1[c]/28	24/27	0/11	11/11
HLA DQ8	26/30	24/27	2/11	4/11
HLA DR3/DR4	3/30	10/27	1/11	1/11

[a] Controls are either 1st degree relatives with no antibodies or HLA matched general population cohort of DAISY/TEDDY.
[b] T1D are recent onset patients within 6 months of diagnosis who were typed for HLA-DQ8.
[c] Antibody-positive control was not removed from the dataset; *p*-values did not differ significantly when data were analyzed with and without this value.

Table 2
Cohort 1 − T cell Responses to defined autoantigens and WE14 TGase treated and untreated.

Antigen	T1D		Control		*p*-value[a]
	Mean SI	±	Mean SI	±	
Tetanus Toxoid	58.1	73.9	61.9	81.1	0.895
Pediacel®	123.4	95.8	145.2	132.1	0.452
P 1	2.5	3.1	0.9	0.8	0.079
B9-23	2.4	2.7	1.1	1.5	**0.047**
GAD 3	2.5	3.7	1.7	1.7	0.441
GAD 4	3.1	3.9	1.8	2.1	0.148
R 2 (IA-2)	2.0	2.4	2.2	2.7	0.873
WE14, 20 μM	2.5	2.3	1.5	1.5	0.086
WE14, 40 μM	2.6	2.7	1.5	1.4	**0.042**
WE14 + TGase, 20 μM[b]	2.8	3	1.3	1.3	**0.005**
WE14 + TGase, 40 μM[b]	3.5	4.2	1.8	1.4	**0.040**
TGase only	1.3	1.4	1.4	1.7	0.680

[a] SI was computed as total spot numbers per analyte/total spot numbers for no antigen. Results were compared using Student's *T* test. Values which were significant are in bold type. Spot numbers for no antigen for type 1 patients were 2.5 ± 2.0 and for control subjects 2.7 ± 3.4.
[b] Due to the TGase-treatment protocol used for cohort 1, the concentrations shown represent theoretical maximal concentrations. Actual concentrations may be significantly lower.

2.5. Autoantibody measurements

IAA, GADA, and ICA512 were determined by the UCD DERC clinical core using established assays [7,8]. ZnT8A were either determined using the "standard" RIA [9], or a modified procedure using a trimeric probe containing sequentially the R, Q, and W variants of the ZnT8 C-terminal domain [10].

2.6. HLA genotyping

HLA genotyping was performed by the UCD DERC clinical core. Individual DRB1 and DQB1 alleles were identified by reverse hybridization of PCR amplicons (18) to either sequence specific oligonucleotide bead arrays (DRB1; Luminex xMAP; One Λ, Canoga Park, CA), or linear arrays (DQB1; Roche Molecular Systems, Alameda, CA), respectively.

2.7. Statistics

The stimulation Index (SI) for each peptide/antigen was computed and then an unpaired two-tailed Student's *T* test was performed for each peptide/antigen within their respective cohort. Significance was defined as <0.05. All data was analyzed using Prism software (GraphPad Software, San Diego, CA).

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