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Original Research Article

High expression of OX40 (CD134) and 4-1BB (CD137) molecules on $CD4^+CD25^{high}$ cells in children with type 1 diabetes

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ABSTRACT

Purpose: Despite the rapidly rising incidence of diabetes in children, with the highest rise in children < 5 years of age, data on mechanisms that trigger severe beta-cells damage are limited. The aim of the study was to assess the frequency of OX40 (CD134) or 4-1BB (CD137) positive cells in the peripheral blood of children with newly diagnosed type 1 diabetes (T1D) in comparison to healthy controls.
Material/methods: The study included 33 children (mean age 7.3 ± 5.4 years) with newly diagnosed T1D and 39 age-matched healthy controls. Separate analysis was performed in children < 5 years. Flow cytometric analysis was performed using the following markers: CD4, CD25, CD137, and CD134. Fasting C-peptide level was assessed as well.
Results: The frequency of $CD4^+CD25^{high}OX40^+$ was higher in T1D children than in controls (median value 3.58% vs. 1.1%, respectively; $p = 0.003$). Moreover, T1D children had higher frequency of $CD4^+CD25^{high}4\text{-}1BB^+$ cells than healthy subjects (median value 5.76% vs. 3.74%, respectively; $p = 0.037$). A significant correlation was noted between the age of diabetic children and the C-peptide level ($r = 0.54$, 95% CI [0.19–0.77], $p = 0.004$). In comparison with age-matched controls, children < 5 years had higher frequency of $CD4^+CD25^{high}OX40^+$ ($p = 0.004$) and $CD4^+CD25^{high}4\text{-}1BB^+$ cells ($p = 0.079$).
Conclusions: Our study showed higher frequency of both OX40 and 4-1BB positive cells in T1D children in comparison to controls. It seems that observed differences might be more pronounced in children < 5 years of age than in older subjects. Further clinical studies are needed to determine the age-related differences in the immune system, in the pathogenesis of T1D.

1. Introduction

Type I diabetes (T1D) is a T-cell-mediated autoimmune disease which occurs as a consequence of the organ-specific immune destruction of the insulin-producing beta cells.

There has been a continuous increase in the incidence of type 1 diabetes in the pediatric population with the highest rise in the youngest children under the age of five [1]. Factors regulating the progress of beta-cell failure and determining time of presentation of clinical diabetes are still poorly understood. Current data indicate that the loss of tolerance to islet autoantigens is a result of imbalance between proinflammatory and anti-inflammatory T cells. A number of studies confirmed that abnormalities of T regulatory cells (Tregs), either in cell number or in function, are associated with initiation and progression of type 1 diabetes [2,3].

Some animal and human studies have found that the tumor necrosis factor receptor (TNFR) family members OX40 (CD134) and 4-1BB (CD137) cells may influence the incidence of type 1 diabetes [4]. Both cells play major roles as costimulatory receptors for CD4 as well as CD8 T cells. The nonobese diabetic (NOD) mouse model used to dissect the mechanisms of lack of immune tolerance and autoimmune T1D confirmed that both the CD4 and CD8 subsets of T cells play a role in the development of disease [5,6]. Both OX40 and 4-1BB signaling activates PI3K/Akt, AP-1, and NF-κB, which promote cell division, survival, and cytokine production. Moreover, OX40 and 4-1BB cells can reduce the suppressor activity of Tregs by direct engagement of receptors on Tregs or by triggering signals on T-cell population [7–9]. It was confirmed that OX40 decreases IL-10 production by Treg cells and it was suggested that OX40 might inhibit the generation of IL-10-producing CD4 regulatory T cells [10].

To our best knowledge, there are no studies evaluating the expression of OX40 and 4-1BB positive T-regulatory cells in children with type 1 diabetes. The aim of the current study was to assess the frequency of $CD4^+CD25^{high}$ cells with expression of OX40 or 4-1BB molecules in the peripheral blood of children with

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newly diagnosed type 1 diabetes, in comparison to age-matched healthy controls.

2. Material and methods

The study group consisted of 72 children treated in the Department of Pediatrics of the Medical University in Warsaw, Poland. The analysis included 33 children (12 girls and 21 boys) of the mean age 7.3 ± 5.4 years (range 0.9–16.5 years), with newly recognized type 1 diabetes mellitus, and 39 controls (19 girls, 20 boys) of the mean age 6.6 ± 4.9 years (1.3–15.9 years). All the participants were Caucasians of Polish origin. The separate analysis was performed in the youngest children under the age of five and in the group of elder subjects. The characteristics of the patients is shown in Table 1.

Type 1 diabetes was diagnosed according to the ISPAD criteria [11]. All children with newly recognized diabetes and presenting symptoms of hyperglycemia had a random serum glucose test showing a result of over 11.1 mmol/l. They were treated with intensive insulin therapy, but no other drugs were administered in this group. Moreover, each subject with type 1 diabetes was positive of at least one β-cell autoantibodies: against glutamic acid decarboxylase (anti-GAD), anti islet cell (anti-ICA), or protein tyrosine phosphatase IA2 (anti-IA2). Median values of antibodies were: in children under 5 years of age (anti-GAD 1.3 U/ml, anti-IA2 5.2 U/ml) and in subjects over 5 years of age (anti-GAD 6.8 U/ml, anti-IA2 5.1 U/ml). Anti-islet cells antibodies were determined by qualitative method; in both groups over 90% of subjects had positive antibodies anti-ICA. Peripheral blood samples were collected from the diabetic patients in the second week of hospitalization, after obtaining metabolic compensation. The control group consisted of children without signs of other autoimmune, chronic, inflammatory or neoplastic diseases. Subjects with fasting blood glucose of over 5.5 mmol/l, as well as personal or family history of type 1 diabetes were excluded. The study was approved by the local ethics committee. Informed consent was obtained from all parents and participants over 16 years old.

2.1. Flow cytometric analyses

Whole blood samples were collected on K-EDTA; mononuclear cells were isolated using a standard Ficoll-Histopaque®-1077 (Sigma Aldrich Co, St. Louis, USA) gradient centrifugation (Sigma Diagnostic Instruction Manual). Flow cytometric analyses were performed using the following monoclonal antibodies purchased from Becton Dickinson: anti-CD4 (PE-Cy-5), anti-CD25 (PE-Cy7), anti-CD137 (FITC) and anti-CD134 (PE). Appropriate isotype control antibodies were used. Samples were evaluated within 4 h on a Cytomics FC500 flow cytometer (Beckman Coulter). A minimum of 10^5 events were acquired for each analysis. The percentages of positive cells were calculated. To determine absolute cell counts, a small volume of blood was analyzed for complete blood count with differential when available. Absolute counts were determined by multiplying the frequency of positive cells determined in the cytometric analysis by the number of lymphocytes $\times 10^3/\mu l$, as determined by complete blood count. No differences were seen between the study groups in any immunological parameters of the peripheral blood mononuclear cells (data not shown). The gating strategy for cells was shown in Fig. 1.

C-peptide immunoradiometric assay (IRMA-CEPEP) was used for determination of C-peptide in serum. The kit was from CIS bio international, Gif-Sur-Yvette Cedex, France, normal range 1.06–3.53 ng/ml.

2.2. Statistical analysis

The results were analyzed with Statistica 8.0 for Windows (StatSoft, Poland) and GraphPad Prism 5.0 (GraphPad Software, USA). The assumption that the data were obtained from populations which follow Gaussian distributions was tested using the Kolmogorov and Smirnov method and Shapiro–Wilk normality test. The comparisons between groups were made with the Student's *t*-test (unpaired, 2-tailed) or, in the case of non-parametric data, with the Mann–Whitney *U*-test. Results were presented as mean values with standard deviations (SD) or for non-parametric data as median values and 25th–75th percentile. The degree of association between two variables was calculated with Spearman's rank correlation coefficient (r). P-values of less than 0.05 were considered as significant.

3. Results

The frequency of $CD4^+CD25^{high}$ cells was higher in children with T1D comparing to controls (median value 3.82% range 2.72–4.88% vs. 1.24% range 0.69–1.6%, respectively, $p = 0.0001$). Children affected by type 1 diabetes under the age of five had lower frequency of these cells than children diagnosed with diabetes over five years of age (0.86% range 0.61–1.28% vs. 1.46% range 1.22–2.38%, respectively, $p = 0.003$). The frequency of $CD4^+CD25^{high}$ cells was significantly lower in T1D subjects under the age of five than in controls (0.86% range 0.61–1.28% vs. 4.09% range 3.38–5.46%, respectively, $p = 0.0001$). Similar difference was noted in children diagnosed with diabetes over the age of five (1.47% range 1.22–2.38% in comparison to healthy controls 3.27% range 2.04–4.66%, $p = 0.003$).

The frequency of $CD4^+CD25^{high}$ cells with expression of OX40 (CD134) was higher in children with T1D than in healthy controls (median value 3.58%, range 1.09–5.34% vs. 1.1% range 0.41–2.83, respectively; $p = 0.003$). Moreover, the age dependent analysis showed that the frequency of these cells was higher in children with T1D diagnosed under the age of five (median value 3.91%, range 0.99–5%), as compared to age-matched healthy controls (median value 0.45%, range 0–1.52%, $p = 0.004$). No statistical difference in these cells was noted between children with diabetes diagnosed at the age of over five, and age-matched controls (median value 2.94, range 1.06–5.75 vs. 1.555 range 0.95–3.43, respectively; $p = 0.286$). There was also no difference in the frequency of $CD4^+CD25^{high}$ cells with expression of OX40 between children with diabetes diagnosed under and over the age of five

Table 1
The characteristics of included patients.

Patients	Number	Gender, F/M	Age (years)	HbA1c (%)
T1D	33	12/21	7.3 ± 5.4 (0.9–16.5)	11.8 ± 2.2
Control	39	19/20	6.6 ± 4.9 (1.3–15.9)	–
T1D < 5 years	19	7/12	2.9 ± 1.1 (0.9–4.9)	11.8 ± 1.8
T1D ≥ 5 years	14	5/9	11.3 ± 3.5 (5–16.5)	11.9 ± 1.8
Control < 5 years	20	9/11	2.6 ± 1.0 (1.3–4.6)	–
Control ≥ 5 years	19	9/10	10.7 ± 3.7 (5.3–15.9)	–

Data shown as mean values with SD.