Human Immunology 76 (2015) 667–672

Contents lists available at ScienceDirect

journal homepage: www.elsevier.com/locate/humimm

Rapid Communication

Altered crosstalk in the dipeptidyl peptidase-4-incretin-immune system in type 1 diabetes: A hypothesis generating pilot study

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ARTICLE INFO

Article history:
Received 13 May 2015
Revised 31 July 2015
Accepted 27 September 2015
Available online 3 October 2015

Keywords:
Type 1 diabetes
GLP1
DPP4
CXCR3
T_{reg}

ABSTRACT

Both $GLP1^{7-36}$ (via GLP1 receptor) and the dipeptidyl peptidase-4 (DPP4) cleaved form of GLP1 ($GLP1^{9-36}$, independently of GLP1R) may modulate the response of lymphocytes to cytokine stimuli. The incretin axis, CXCR3 (receptor of DPP4 ligand cytokines CXCL9–11) expression on T_{reg}s and hematologic parameters were assessed in 34 patients with long standing type 1 diabetes (T1DM) and in 35 healthy controls. Serum DPP4 (sDPP4) activity, plasma total GLP1 and $GLP1^{7-36}$ concentrations were determined. $GLP1^{9-36}$ concentrations were calculated. CXCR3 expression (flow cytometry) was higher on the $CD25^{-/low}Foxp3^{+}$ than on the $CD25^{+}Foxp3^{+}$ T_{reg}s independently from T1DM, suggesting that $CD25^{-/low}Foxp3^{+}$ T_{reg}s are possibly waiting for orientational chemotactic stimuli in a "standby mode". The higher sDPP4 activities in T1DM were inversely correlated with $GLP1^{7-36}$ levels and $GLP1^{9-36}$ levels directly with lymphocyte counts in controls. Our results might indicate an altered DPP4-incretin system and altered immunoregulation including a potentially dysfunctional $GLP1^{9-36}$ signaling in T1DM.

1. Introduction

Besides the regulation of pancreatic endocrine function the incretin axis is involved in the regulation of immune responses. In vitro glucagon-like peptide-1 (GLP1) was found to enhance the polarization of human macrophages via STAT3 signaling toward M2 phenotype which reduce T_h1 responses via IL-10, TGF-β and IL-1 receptor antagonist secretion and promote angiogenesis and tissue remodeling [1]. GLP1 receptor (GLP1R) was described on T-cells [2] and invariant natural killer (iNKT) cells [3] and the role of GLP1R signaling was indicated in the maintenance of the peripheral regulatory T cell (T_{reg}) population [4]. After exenatide (GLP1R agonist) treatment of type 2 diabetic (T2DM) patients with psoriasis the improvement of skin symptoms was reported, while the numbers of iNKT cells increased in the circulation, but decreased in psoriatic plaques and a dose dependent inhibition of their cytokine secretion was also observed [3]. Beneficial effect of the DPP4 inhibitor sitagliptin was also reported in severe psoriasis [5]. Increased serum DPP 4 activity was reported to play a role in the development of insulin resistance in metabolic syndrome and T2DM [6] which has a well established link with psoriasis [7]. The linkage of T1DM and psoriasis is less clear, however, a few authors reported a high prevalence (up to 9%) of psoriatic skin lesions in T1DM [8] compared to approximately 2% in the general population [9].

There are additional observations relating the DPP4-incretin axis to immunoregulation: numerous cytokines are substrates of DPP4 including the major eosinophil chemoattractant CCL11 and in addition CXCL10 and 12 which were described to play an important role in T-lymphocyte targeting and homing [10–12]. $GLP1^{9-36}$ was described to inhibit the chemokine-induced migration of $CD4^{+}$ lymphocytes independently of GLP1R signaling [13]. CXCR3 (the

Abbreviations: BAL, bronchoalveolar lavage; CCL, chemokine (C-C motif) ligand; CNTRL, control; CST, Chi-square test; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; CXCL, chemokine (C-X-C motif) ligand; CXCR3, C-X-C chemokine receptor type 3; DPP4, dipeptidyl peptidase-4; ELISA, enzyme-linked immunosorbent assay; Foxp3, forkhead box P3; GADA, glutamic acid decarboxylase autoantibody; GLP1, glucagon-like peptide 1; GLP1R, GLP1 receptor; ICA, islet cell autoantibody; IL, interleukin; HbA1C, glycated hemoglobin; iNKT cell, invariant natural killer T-cell; MWU, Mann–Whitney *U*-test; NOD, non-obese diabetic; SRO, Spearman Rank-Order test; T1DM, type 1 diabetes mellitus; T_h, helper T-cell.

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http://dx.doi.org/10.1016/j.humimm.2015.09.018
0198-8859/

receptor of CXCL9–11) signaling was indicated to be crucial in targeting T_{reg} cells to pancreatic islets in NOD mice [14] and recent-onset diabetes in NOD mice could be reversed by DPP4 inhibitor treatment, that also resulted in the increased number of T_{reg} cells in pancreatic lymph nodes [15]. The upregulation of DPP4 expression was described in the airway epithelial of asthmatic patients and in vitro the IL13 (T_h2 type cytokine) treatment resulted in the upregulation of DPP4 on the mRNA level in airway epithelial cells [16]. Sitagliptin treatment markedly decreased leukocyte accumulation as well as IL13 levels in the BAL fluid and ovalbumin specific immunoglobulin-E in serum of ovalbumin-induced murine model of allergic airway disease [17]. Although atopic disorders including asthma bronchiale are theoretically considered to be inversely related to T1DM due to the difference in T_h1/T_h2 imbalance, the increase in the incidence of both atopic and also autoimmune disorders may possibly be explained in part by the 'hygiene hypothesis'.

Our study group previously reported elevated serum DPP4 enzymatic activity [18] and the extension of the $CD4^+Foxp3^+CD25^{-/low}$ T_{reg} ("incomplete/reserve" T_{reg} [19]) population [20] in T1DM patients. The CTLA4 expression is decreased in $CD4^+Foxp3^+CD25^{-/low}$ T_{reg} cells [20] and the suppressive capacity of this cell subpopulation is also reduced [21]. Although the reported beneficial effect of DPP4 inhibitors and incretin mimetics in atopic and autoimmune diseases and also T1DM [22] indicate a significant role of the DPP4-incretin axis in immune regulation the details are still elusive. Our aim was to find additional changes in the immune network that may contribute to the explanation of these previous findings and may potentially link these observations together. Due to that CXCR3 deficient NOD mice develop diabetes in a more accelerated manner likely due to the impaired targeting of T_{reg} cells [14] we aimed to measure the T_{reg} CXCR3 expression in our patients.

2. Methods

2.1. Patients and study setup

The study protocol was previously approved by the Semmelweis University Regional and Institutional Committee of Science and Research Ethics and all volunteers gave written informed consent. 34 T1DM patients (F/M = 18/16) treated at the outpatient clinics of our department and 35 age, gender and BMI matched non-diabetic control subjects (F/M = 19/16) without a family history of T1DM were enrolled on a voluntary basis. T1DM patients were diagnosed according to international standard criteria [23] and had a mean duration of 15 years after the diagnosis. The T1DM patients and controls participated in a study published earlier and this study is the extension of the previously report [20]. Patients with any suspect of hepatic disease or on systemic immunomodulatory medication were excluded from both study groups. Control subjects were free of known autoimmune and endocrine disorders. T1DM subjects with extreme disturbances of carbohydrate metabolism (HbA1C above 12% and/or fasting plasma glucose above 20 mmol/l) were also excluded (see clinical characteristics in Table 1).

2.2. Assessment of serum DPP4 enzymatic activity and plasma GLP1 levels

Serum DPP4 activity was determined in a continuous monitoring assay in a Varioskan Flash microplate reader (Thermo Scientific, Waltham, MA, USA) at 405 nm, 37 °C for 30 min, using 9.4 µl serum and 115.6 µl assay buffer (10 mM Tris–HCl, pH 7.6) containing 2 mmol/L Gly-Pro-paranitroanilide tosylate substrate (Bachem, Bubendorf, Switzerland) in each microplate well. Enzyme activity is expressed in nmol/ml/min (U/L) of pNA hydrolyzed. Active ($GLP1^{7-36}$ amide) and total GLP1 ($GLP1^{7-36}$ amide and $GLP1^{9-36}$ amide) were assessed from 45 min postprandial plasma samples after a mixed meal containing 50 g carbohydrate, 22 g protein and 9 g fat. Measurements were made using specific ELISA Kits according to the manufacturers recommendations (EMD Millipore, Billerica, MA, USA). Plasma levels of the cleaved form of GLP1 were calculated from the total and $GLP1^{7-36}$ concentrations. Plasma samples for GLP1 assessment were treated with sitagliptin and a protease inhibitor cocktail (P8340, Sigma Aldrich, Saint Luis, MO, USA). All measurements were made in duplicates.

2.3. Additional recorded data

Routine hematologic parameters were assessed using a Siemens ADVIA 2120 Hematology System (Erlangen, Germany) according to standard methods. Plasma glucose concentrations were assessed with a Beckman Coulter AU5800 Clinical Chemistry System (Brea, CA, USA). C-peptide measurements were made using a Liaison XL chemiluminescence analyzer (Dia Sorin, Saluggia, Italy). HbA1c was measured with a Bio-Rad Variant II Turbo Hemoglobin Testing System (Hercules, California, USA) according to the Approved IFCC reference method. Glutamic acid decarboxylase autoantibody (GADA) serum levels were determined by Euroimmun Anti-Glutamic Acid Decarboxylase (GAD) ELISA (IgG) Kit (Euroimmun AG, Luebeck, Germany). ICA (islet cell autoantibody) was measured with indirect immunofluorescence on monkey pancreas tissue (Inova Diagnostics, San Diego, CA, USA).

2.4. Flow cytometric analysis

Fasting, EDTA-anticoagulated blood samples were collected. The flow cytometric analysis was performed as described in detail in our prior publication [20] using the following flow cytometric antibodies: CD3-PE-Cy7, CD8-PerCP, CD25-AF700 (Biolegend, San Diego, CA, USA), Foxp3-PE-CF594 (BD Biosciences, San Jose, CA, USA). In addition the CXCR3 surface protein measurements (Fluorescein-labeled antibody was purchased from R&D Systems, Minneapolis, MN, USA) from the flow cytometric data has also been analyzed in the present study. A Beckman Coulter Navios flow cytometer and 1.2 version of Beckman Coulter Kaluza software (Brea, CA, USA) were used for quantitative analysis. A clear discrimination of $CXCR3^+$ cells allowed to calculate the proportion of $CXCR3^+$ and $CXCR3^{neg}$ cells in both types of T_{reg} subpopulations ($Foxp3^+CD25^+$ and $Foxp3^+CD25^{-/low}$). Our gating strategy is described on Fig. 1.

2.5. Statistical analysis

Statistica software (version 12, StatSoft, Tulsa, OK, USA) was used. Kolmogorov–Smirnov test was used to assess normality. Logarithmic transformation was used for GLP1 plasma levels and DPP4 serum activities in order to gain normal data distribution. In cases of normal distribution unpaired two-tailed *T*-test, in cases of non-normal distribution Mann–Whitney *U*-test (MWU) were used to compare means in different study groups. Spearman Rank-Order (SRO) and Pearson tests were used to assess correlations. Statistical power was calculated using correlation coefficient *z*-transformation. Chi-square test (CST) was used to assess gender proportion similarity. Wilcoxon test was used to compare dependent variables.

3. Results

3.1. Serum DPP4 activity and plasma GLP1 levels

We found higher DPP4 enzymatic activity (U/L) in the sera of T1DM patients compared to the controls (T1DM: 44.13 [95% CI: