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RESEARCH

Higher Serum DPP-4 Enzyme Activity and Decreased Lymphocyte CD26 Expression in Type 1 Diabetes

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Abstract Dipeptidyl peptidase-4 (DPP-4) is involved in the metabolism of peptide hormones, T-cell activation and proliferation. In type 1 diabetes mellitus (T1DM) β-cell destruction involves a number of dysregulated T-cells. Our aim was to assess the serum DPP-4 activity and the lymphocyte membrane bound CD26 expression in patients with type 1 diabetes and healthy controls. Ninety-eight (T1DM: 48, F/M=20/28, mean age: 34.4y; control: 50, F/M=39/11 mean age: 32,4y) individuals were included. Fasting serum DPP-4 enzymatic activity, plasma glucose (FPG), CD26 expression on CD3+, CD4+ and CD8+ lymphocytes, HbA1c and body mass index (BMI) were assessed. ICA and GAD antibodies were assessed in the T1DM group. DPP-4 enzymatic activity was determined by kinetic enzyme assay, ICA and GAD were assessed by ELISA. Determination of the CD26 expression on CD3+, CD4+ and CD8+ lymphocytes was performed by flow-cytometric analysis. We found higher serum DPP-4 activity (Mean: T1DM: 30.069 U/L, control: 22.62 U/L, $p<0.0001$) and decreased CD26 lymphocyte expression on all lymphocyte subpopulations in T1DM. Fasting serum DPP-4 activity was independent from the ICA or GAD status of patients with T1DM. Here we first present that the serum DPP-4 activity is increased and the lymphocyte membrane bound CD26 expression is decreased in type 1 diabetes. Decreased lymphocyte membrane bound CD26 expression in T1DM might be a novel part of the T-lymphocyte regulatory dysfunction observed in type 1 diabetes mellitus. These results might provide some basis for the clinical implication of DPP-4 inhibition in patients with T1DM.

Keywords Dipeptidyl peptidase-4 (DPP-4) · CD26 · Type 1 diabetes (T1DM) · Islet cell antibody (ICA) · Glutamic acid decarboxylase antibody (GAD)

Abbreviations

DPP-4 CD26	Dipeptidyl peptidase-4
ICA	Islet cell antibody
GAD	Glutamic acid decarboxylase antibody
GLP-1	Glucagon like peptide-1
HbA1C	Glycated haemoglobin
BMI	Body mass index
FPG	Fasting plasma glucose

Introduction

Dipeptidyl peptidase-4 (DPP-4, CD26) is an abundantly distributed serin protease which is enzymatically active in both soluble and membrane bound forms. DPP-4 regulates various physiological processes by cleaving peptides in the

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circulation, including many chemokines, growth factors, neuropeptides and peptide hormones. With the degradation of incretin hormones, DPP-4 plays an important role in carbohydrate metabolism [1].

In addition to the control of glucose homeostasis through the regulation incretin hormone system, DPP-4 has several other biological activities. It has been implicated in immunological functions such as the T cell costimulatory function of the membrane bound CD26, cell migration, inflammation and also in cancer metastasis. Both the membrane bound and the soluble DPP-4 is active in a dimer form. Membrane bound CD26 is expressed in many different cells and tissues, including melanocytes, epithelia of the renal tubule or colonic mucosa, endothelial cells and T lymphocytes. Both the soluble and the membrane bound form of CD26 can modulate in vitro T cell proliferation and has a role in T cell immune responses [1, 2]. DPP-4 is expressed at low levels on resting T cells, however, DPP-4 expression increases following T cell activation [3, 4]. Additionally, caveolin-1 on the APC ligates CD26 dimers on the T cell surface, resulting in the recruitment of lipid rafts in the plasma membrane and the recruitment of CARMA1 to the cytosolic portion of CD26. Ultimately, these steps lead to the activation of NF-kB, to T cell proliferation, and to IL-2 production [5, 6]. It has costimulatory function that enhances antigen-specific T cell activation, while the soluble form of DPP-4 enhances the T cell transendothelial migration in vitro [3, 4]. In general the increase in sCD26 (DPP-4) enzyme activity did not reflect systemic immune activation, nevertheless in certain clinical settings increased DPP-4 activity suggested to indicate T cell activation in the liver such as in HIV–HCV–coinfected patients with hepatotoxicity after HAART [7].

Several observations were published on the serum DPP-4 activity and the membrane bound CD26 expression in patients with different diseases. The increase of the serum enzymatic activity in non-alcoholic fatty liver disease, multiple sclerosis, Graves' disease, Hashimoto thyroiditis, sarcoidosis, primary biliary cirrhosis as well as the decrease of activity in ANCA-associated vasculitides and systemic lupus erythematodes was observed [8–13]. Increased CD26 expression was found in allergic asthma, rheumatoid arthritis, inflammatory bowel disease, decreased CD26 expression was reported in HIV infection and T cell lymphoproliferative disorders [14, 15].

Although both the soluble and the membrane bound DPP-4 was studied in several autoimmune diseases, little data is available on the role of DPP-4 in type 1 diabetes mellitus. Therefore, the soluble DPP-4 enzyme activity in the human sera and the CD26 expression on T-lymphocytes were assessed in patients with type 1 diabetes mellitus and in healthy individuals in this study. Islet cell (ICA) and glutamic acid decarboxylase (GAD) antibodies were also determined according to the autoimmune character of the disease.

Methods

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. All the control patients were without any drug treatment. Patients with type 1 diabetes were not taking any gliptins or metformin as adjuvant treatment which may affect the DPP-4 activity.

Individuals with pregnancy, serious underlying disease in the history, actual or chronic drug use, abnormal laboratory values (liver tests, creatinin values, whole blood cell counts, sedimentation, CRP, urine analysis, TSH, ICA, GAD, fasting plasma glucose, HbA1c) were excluded from the healthy control group. Blood samples were collected from each volunteer in fasting state in the morning.

Patients, Clinical Data and Experimental Setup

Forty-eight (F/M=20/28) patients with T1DM, and 50 (F/M=39/11) healthy volunteers (CNTRL) were included in the study. Islet cell antibodies and glutamic acid decarboxylase antibodies were determined in all the 48 patients with type 1 diabetes. Fasting serum DPP-4 activity, fasting plasma glucose (FPG), HbA1c and body mass index (BMI) were assessed in all the 98 (T1DM + CNTRL) individuals (Table 1).

Our previous pilot study has not established a possible direct effect of hyperglycaemia on the serum DPP-4 enzymatic activity therefore in this study a novel comparison has been performed with higher patient population between the T1DM group and 82 patients with type 2 diabetes (F/M=48/34) but without clinically obvious liver disease [16, 17].

ICA and GAD were determined in all the 48 individuals with type 1 diabetes. CD26 expression on CD3+ T lymphocytes and on CD4+, CD8+ T lymphocyte subpopulations were assessed in all individuals.

Kinetic Assay for Fasting Serum DPP-4 Enzymatic Measurement, CD26 Expression and ICA, GAD Antibody Determination

DPP-4 Enzymatic Activity Determination by Kinetic Assay

Serum DPP-4 activity was determined in a continuous monitoring assay in a microplate reader (Multiscan EX Labsystems) at 405 nm, 25°C for 30 min. under similar conditions as described for neutral endopeptidase [18]. All

Table 1 Clinical characteristics and the fasting serum DPP-4 activities of healthy individuals (controls, CNTRL), patients with type 2 diabetes mellitus without liver disease (T2DM) and patients with type 1 diabetes mellitus (T1DM). Mean values are indicated in bold numbers and 95% Confidence Intervals are indicated in parenthesis

	Control	Type 2 diabetes[a]	Type 1 diabetes
Number of individuals (F/M)	**50** (F/M=39/11)	**82** (F/M=48/34)	**48** (F/M=20/28)
Age (years)	**32,4** 95% CI:22–56	**62,8**♦ 95% CI:60,42–65,1	**34,4**• 95% CI:20–60
HbA1C (%)	**5,5** 95% CI:4,9–5,9	**7,53**♦ 95% CI:7,18–7,88	**7,6*** 95% CI:5,2–11,0
Fasting Plasma Glucose (mmol/L)	**4,4** 95% CI:3,3–5,3	**8,2**♦ 95% CI:7,51–8,87	**8,9*** 95% CI:2,3–19,7
Fasting serum DPP-4 (U/L)	**22,62** 95%CI: 16,32–28,28	**23,97** 95% CI:22,32–25,61	**30,06***• 95%CI:21,85–45,94
BMI (kg/m2)	**22** 95%CI: 18,3–26,	**29.77**♦ 95% CI: 28.58–30.96	**24,3****• 95%CI: 19,9–32

[a] In this study patients with type 2 diabetes mellitus were classified to the T2DM group provided that liver disease (NAFLD) has been excluded due to the conclusion that in insulin resistant patients with non-alcoholic fatty liver the serum DPP-4 activity is increased and might be considered as a novel liver disease biomarker (ref. [16])

p values, using one sided Anova test:

* T1DM vs CNTRL, $p<0,01$

** T1DM vs CNTRL, $p<0,05$

♦ T2DM vs CNTRL, $p<0,01$

• T2DM vs T1DM, $p<0,01$

DPP-4 assays were run in duplicates. Fifteen μl serum and 185 μl assay buffer (10 mM Tris–HCl, pH 7.6) containing 4 mmol/L substrate Gly-Pro-pnitroanilide tosylate, Gly-Pro-PNA, (Bachem,Bubendorf, Switzerland) were pipetted into each microplate wells. Enzyme activity was expressed in nmol/ml/min (U/L) of pNA hydrolysed [11].

Elisa Kit for ICA and GAD Determination

ICA and GAD were determined by Medizym ICA screen and anti GAD ELISA kit (Medipan GmBH) in all the 48 individuals with type 1 diabetes.

Flow Cytometric Analysis for CD26 Expression on Lymphocytes

Flow cytometric analyses were performed on peripheral blood. Specimens were subjected to FACS Lysing Solution treatment (BD Biosciences, San Jose, CA) to eliminate mature red blood cells. A 3-color staining was applied for all samples using the following fluorochrome-conjugated antibodies: CD26-PE (BD Biosciences), CD8-APC (BD Biosciences), CD4 PE-Cy5 (DAKO, Glostrup, Denmark) and CD3 FITC (BD Biosciences). All used antibodies were pre-titrated. Stained cells were applied on a bench-top flow cytometer (FACSCalibur, BD Biosciences) and analyzed by using Cell Quest Pro software (BD Biosciences). The instrument settings and fluorescence compensations were regularly controlled by caliBRITE beads (BD Biosciences) and stained cells (e.g., CD4–CD8 stained cells). Twenty-thousand events were measured from all samples. The analyses were performed on isotype control and stained (CD3-CD26-CD4-CD8 stained) sample from each patient.

Cell populations of interest were gated using side scatter (SSC)—forward scatter (FSC) and CD26-CD3 or CD26-CD4 or CD26-CD8 dot plots. The mean fluorescence intensity (MFI) of CD26 positive population was calculated by using Cell Quest Pro software.

Measurement of FPG, HbA1c and C-peptid Parameters

Routine clinical chemistries were assessed using an Olympus AU2700 autoanalyser at 37°C according to standard methods.

Statistical Analysis

The distribution of data was assessed by the Jarque-Bera test. Because we found normal distribution, two-tailed *T*-test, one-sided Anova and Pearson correlations were used to compare means and assess correlations. *P*-values lower than 0.05 were evaluated as significant. For determining the diagnostic utility of the DPP-4 enzyme activity, a set of ROC analyses were performed.

Results

Although the elapsed years from the diagnosis of diabetes seem to be a long period when the autoimmune process itself probably is not very active, autoantibody positivity still occurs in the T1DM group. Auto-antibody ICA was

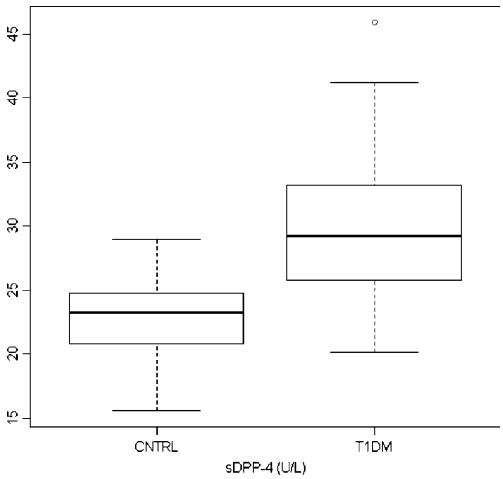

Fig. 1 Boxplot analysis of the serum DPP-4 enzyme activity in the T1DM and in the control (CNTRL) groups. Serum DPP-4 activity is significantly increased in the T1DM group (Anova p=8.75e-12). Results are expressed in U/L. Median values are presented

positive in 41, GAD antibody was positive in 29 cases. Twenty-seven patients were positive for both antibodies.

We experienced a significant increase in the fasting serum DPP-4 enzyme activity in type 1 diabetes 13.4 years (±9.76y) after the diagnosis of the disease (Mean = 30,06 U/L, 95%CI:21,85–45,94 U/L) compared to healthy individuals (22,62 U/L, 95%CI: 16.32–28,28 U/L, two-tailed T-test p<0.0001) (Fig. 1).

The higher serum DPP-4 activity was not different in the sub-groups of T1DM depending on the presence of autoantibodies: 41 ICA positive (Mean = 29.2 U/L, 95%CI:27.59–30.81 U/L) and 7 ICA negative (29.9 U/L, 95%CI:23.31–36.50 U/L) patients, 29 GAD positive (28.66 U/L, 95% CI:26.91–30.41 U/L) and 19 GAD negative (29.44 U/L, 95% CI:26.59–32.28 U/L) patients with type 1 diabetes. There was no alteration in the DPP-4 activity in the 5 ICA and GAD double negative patients (31.14 U/L, 95%CI:22.67–39.61 U/L) compared to those occurred with at least one positive antibody.

The best cutoff point of DPP-4 activity as a single marker determined by ROC analysis was: 25.91 U/L, however due to the sensitivity was 73.1% and the specificity was 83.0% as a single marker. Subsequent ROC analysis was performed to assess whether the addition of serum DPP-4 measurement might increase the accuracy of the autoantibody determination in this set of patients with type 1 diabetes mellitus. We found that the measurement of the serum DPP-4 enzymatic activity increased the sensitivity of the ICA + GAD tests from 89.4% up to 95.7% in patients with type 1 diabetes mellitus 13.4 years (±9.76y) after the diagnosis of the disease (Fig. 2).

CD26 expression was significantly decreased in the diabetes group on all assessed lymphocyte sub-populations (Fig. 3). The CD26 expression:

- on CD3+ lymphocytes: T1DM: 100.32 Mean Fluorescent Intensity (MFI), 95%CI: 92.91–107.72 vs. CNTRL: 119.82MFI, 107.81–131.84, (Anova p=0.0011).
- on CD4+ lymphocytes: T1DM: 89.29MFI, 83.45–95.13 vs. CNTRL: 106.48MFI, 94.97–117.99, (Anova p=0.03294).
- on CD8+ lymphocytes: T1DM: 110.75MFI, 98.42–123.08 vs. CNTRL: 136.45MFI, 121.22–151.68 (Anova p=0.004781).

We found no correlation between the DPP-4 activity and the CD26 expression on any lymphocyte sub-population in any of the groups studied. No correlation was found between the serum DPP-4 enzymatic activity and the CD26 expression within any of the subgroups either.

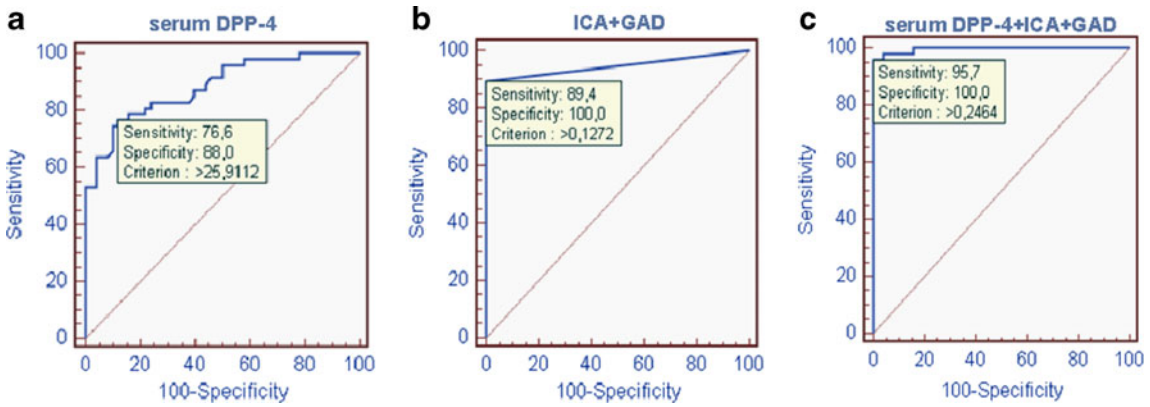

Fig. 2 Receiver Operator Curves (and the most important corresponding data) for **a** Serum DPP-4 activity as a single test for type 1 diabetes (best cutoff point: 25.91 U/L). **b** ICA and GAD autoantibodies as a combined diagnostic test for type 1 diabetes. **c** ICA, GAD and serum DPP activity as a combined diagnostic test for type 1 diabetes. The measurement of the serum DPP-4 enzymatic activity increased the sensitivity of the ICA + GAD tests from 89.4% up to 95.7% in patients with type 1 diabetes mellitus 13.4 years (±9.76y) after the onset (diagnosis) of the disease

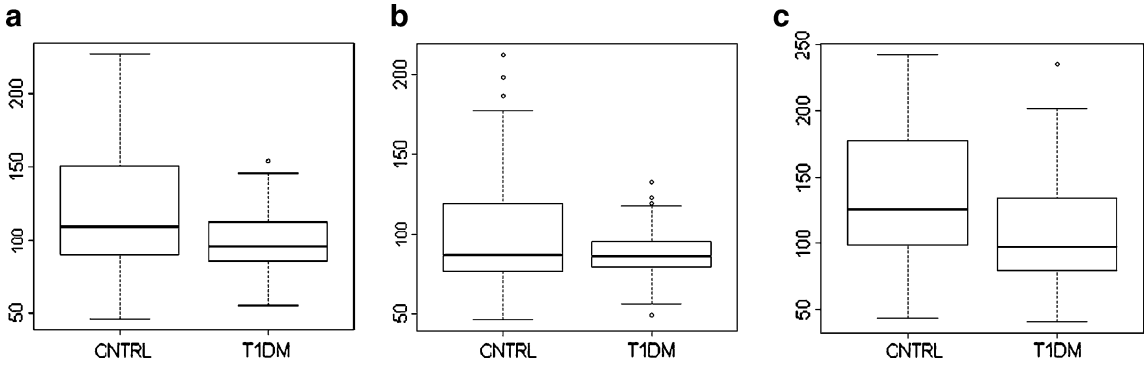

Fig. 3 Boxplot analysis (medians are indicated) for CD26 expression on the CD3+, CD4+, CD8+ T lymphocytes in T1DM. Results are expressed in mean fluorescent intensity (MFI). **a** Decreased CD26 expression on the CD3+ lymphocytes in T1DM (Anova p=0.001154) (Jarque-Bera normality test p-value: CNTRL: 0.0646, T1DM: 0.3986). **b** Decreased CD 26 expression on the CD4+ lymphocytes in T1DM (Anova p=0.03294) (Jarque-Bera normality test p-value: CNTRL: 0.0147, T1DM: 0.5). **c** Decreased CD 26 expression on the CD8+ lymphocytes in T1DM (Anova p=0.00478) (Jarque-Bera normality test p-value: CNTRL: 0.1216, T1DM: 0.0641)

We found no correlation among the DPP-4 activity and other parameters (HbA1c, FPG, BMI, age, time from diabetes onset) tested in this study.

Discussion

The clinical importance of this observation on one hand may be demonstrated by a recently presented pilot study in which sitagliptin reduced total insulin dose, mean blood glucose and HbA1c in patients with T1DM [19].

We found that the measurement of the serum DPP-4 enzymatic activity increased the sensitivity of the ICA + GAD tests from 89.4% up to 95.7% in patients with type 1 diabetes mellitus 13.4 years (±9.76y) after the diagnosis of the disease, when the autoimmune process has burnt out already (Fig. 2). The lower than 90% sensitivity (combined) of the ICA + GAD autoantibody determination might be explained that the mean elapsed time from the establishment of the diagnosis was 13.4 years (±9.76y) in our patient population. The recently published results [16, 17] of our study group showed that surprisingly the serum DPP-4 activity was not increased in T2DM provided that patients with clinically obvious liver disease were intentionally excluded from the study group. We eventually concluded that it is the presence of the (fatty) liver disease that has a primary impact on the serum DPP-4 enzymatic activity and not the directly the hyperglycaemia alone.

Based on the lack of correlations between the serum DPP-4 activity and FPG and also between the serum DPP-4 activity and HbA1c levels the increased DPP-4 enzyme activity should be explained by other reasons such as the autoimmune process itself, a possible hormonal feedback mechanism or theoretically a target organ including the endothelial damage or even an unknown chronic viral infection [11, 20].

In addition to the fact that a recent study established that GLP-1R signaling may regulate lymphocyte proliferation and maintenance of peripheral regulatory T cells [21], our observation that the serum DPP-4 is increased and the T-lymphocyte membrane bound CD26 expression is decreased in patients with T1DM provided further evidence that the entero-insular axis might be altered in this autoimmune disease. Our observation that CD26 expression is downregulated on all studied lymphocyte subpopulations in patients with T1DM might be a novel part of the T-lymphocyte regulatory dysfunction observed in type 1 diabetes mellitus [6, 22]. Caveolin-1 is a putative ligand for CD26 and caspase recruitment domain family, member 11 (CARD11, CARMA1) might emerge as a potential downstream target in CD26 signalling and T cell costimulation [6]. Clinical evidence was found for the correlation between the CD26 expression and disease severity of multiple sclerosis and rheumatoid arthritis suggesting a potential role of CD26 mediated T cell costimulation in these autoimmune diseases [23, 24]. The potential role and the significance of alterations of the entero-insular axis in T1DM is further actualized by the observation that DPP-4 inhibitor treatment demonstrated beta-cell protective properties in certain experimental animal studies in type 1 diabetes models [24].

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Competing interests The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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