

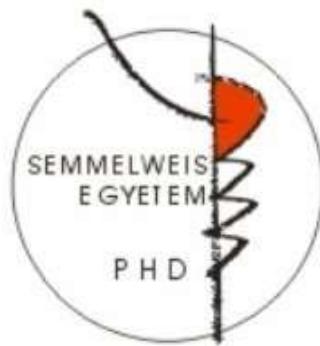
Serum dipeptidyl-peptidase-4 enzyme activity and T-lymphocyte surface CD26 expression in diabetes mellitus

Short Doctoral thesis

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Introduction

Dipeptidyl peptidase-4 (DPP-4) enzyme occurs in soluble and membrane-bound (CD26) forms in the human body. Both forms have T-cell activation and T-cell proliferation-increasing effects as well as DPP-4 activity, therefore they cleave and thus inactivate the incretin hormones secreted following carbohydrate intake. The DPP-4 inhibitors based on the inhibition of the DPP-4 enzyme and currently applied in type 2 diabetes (T2DM) take advantage primarily of the blocking of the enzyme's effect on incretins in diabetes treatment. However, the protein has been studied in relation to several diseases. Based on the variances of its activity, the expression of the membrane-bound form, it has become evident that it has a fundamental role, *inter alia*, in inflammatory, autoimmune and tumorous processes alike.

Research so far has not paid significant attention to the examination of enzyme activity in type 1 diabetes (T1DM). At the same time, in this autoimmune disease involving carbohydrate metabolism disorder, a more rigorous and detailed examination of the molecules that play a role in the activity of the immune system as well can be especially important regarding the pathomechanism, screening and possible prevention of the disease alike. In the light of previous data in the literature, we presumed that the status of the carbohydrate metabolism may be characteristic of and the current hyperglycemia may be decisive of the DPP-4 enzyme activity values measured in type 1 diabetic patients and that serum DPP-4 enzyme activity may vary due to carbohydrate intake. Part of our study is focused on this process.

It was our hypothesis, furthermore, that there may be an interrelation between autoimmune-based diabetes and the functioning of the DPP-4 protein, whose verification, in turn, could lead to the exploration of additional information on the pathomechanism of the disease and might enhance our current knowledge of its diagnosis and treatment. Taking into consideration the autoimmune nature of T1DM, the role of DPP-4/CD26 in immunological processes and the fact that the role of soluble DPP-4/sCD26 and its connection to membrane-bound CD26 has not been clarified entirely, we also assumed a correlation between the different forms of manifestation of the two basically identical parameters (sDPP-4 and lymphocyte surface-bound CD26).

Objectives

1. To determine fasting and postprandial serum DPP-4 enzyme activity in type 1 and type 2 diabetic patients as well as in healthy individuals.
2. To examine whether soluble DPP-4 activity measurable in serum undergoes change upon carbohydrate intake in healthy individuals on the one hand and diabetic patients on the other.
3. Is there any correlation between fasting serum DPP-4 enzyme activity and the clinical laboratory values related to the carbohydrate metabolism (fasting plasma glucose, HbA1C) in T1DM and T2DM patients on the one hand and healthy individuals on the other?
4. Examination of the background of elevated serum DPP-4 activity detected in T1DM in view of the findings of the examination.

Given the autoimmune nature of T1DM and the role of DPP-4/CD26 in immunological processes, in addition to determining the fasting serum DPP-4, our goal was to determine the expression of CD26 bound to the surface of the T-lymphocytes taking part in the immunological processes, which, at the same time, plays a role in T-cell activation and proliferation, in CD3+ lymphocyte and subsequently CD4+ and CD8+ lymphocyte subpopulations in T1DM on the one hand and healthy individuals on the other.

5. To examine whether there is any correlation between fasting soluble serum DPP-4 activity and lymphocyte membrane-associated CD26 expression in autoimmune diabetic patients.
6. To identify ICA and GADA markers from among the island-cell antibodies appearing as markers of the autoimmune process in T1DM. Examination in T1DM of whether there is any correlation

- between the fasting serum DPP-4 enzyme activity values measured and the autoimmune markers examined in autoimmune diabetic patients,
 - between the autoimmune markers examined on the one hand and the CD4+ and CD8+ T lymphocyte subpopulations' surface CD26 molecule expression on the other in autoimmune diabetic patients.
7. In the light of the findings, to determine the possible role of the DPP-4 enzyme in type 1 diabetes.

Patients and methods

Our patients consisted of type 1 and type 2 diabetic patients on the one hand and healthy individuals (CNTRL) on the other.

I. Examination focusing on determining fasting and postprandial serum DPP-4 enzyme activity

We examined the serum DPP-4 activity in type 1 diabetic patients in fasting and postprandial states. The control group consisted of healthy individuals and we choose type 2 diabetic patients to form a hyperglycemic control group.

A total of 153 people participated in the examination, T1DM and T2DM diabetic patients as well as healthy individuals in the following breakdown: 41 T1DM (female/male=17/24; age: $36,39 \pm 12,03$ years; BMI: $25,25 \pm 4,33$ kg/m²), 87 T2DM (female/male=47/40; age: $62,96 \pm 11,10$ years; BMI: $29,49 \pm 5,20$ kg/m²) and 25 healthy individuals (female/male=15/10; age: $35,48 \pm 13,99$ years; BMI: $23,24 \pm 3,89$ kg/m²).

We determined fasting and- following the test food intake-postprandial serum DPP-4 enzyme activity variation in the subjects examined. During the analysis, in the course of the examination of postprandial serum DPP-4 activity, determination of DPP-4 enzyme activity from the serum took place at various points in time in the case of 50 individuals (17 T2DM, 15 T1DM patients and 18 healthy individuals) at both 60 and 120 minutes following the test food intake (50g carbohydrate+24g protein+12g fat = 410 kcal). For all 153 individuals, in addition to fasting serum DPP-4 enzyme activity, clinical laboratory values (fasting plasma glucose, HbA1C, GOT, GPT, GGT, ALP, creatinine, complete blood count, CRP) were also determined.

II. Examination focusing on the background of elevated serum DPP-4 activity

In view of the results of the previous analysis – higher serum DPP-4 activity detected in the T1DM group – we carried out further tests focusing on exploring the background of the findings. In the rest of the examination we tested T1DM patients and healthy individuals with

the participation of a total of 98 people in the following breakdown: 48 T1DM patients: (female/male=20/28; average age: 34,4 95% CI:20-60 years; BMI: 24,3 95%CI: 19,9-32 kg/m²) and 50 healthy control individuals: (female/male = 39/11; average age: 32,4 95% CI:22-56 years; BMI: 22 95%CI: 18,3-26 kg/m²).

In the course of the examination of the background of elevated serum DPP-4 activity, in addition to determining clinical laboratory values (fasting plasma glucose, HbA1C, GOT, GPT, GGT, ALP, creatinine, CRP, C-peptide and complete blood count) soluble serum DPP-4 activity and membrane-associated DPP-4, that is, CD26 expression were also determined in all cases both in T1DM patients and healthy individuals. We examined membrane-associated CD26 expression in CD3+ T lymphocytes and subsequently both in CD4+ and CD8+ T lymphocyte subpopulations. We also demonstrated the presence of ICA and GADA markers in the T1DM group from among the island-cell antibodies appearing as markers of the autoimmune process in T1DM.

The time elapsed since making the diagnosis of diabetes in T1DM patients is 13.4±9.76 years on average.

Determination of clinical laboratory parameters

Determination of laboratory parameters from the blood samples taken took place in accordance with standard laboratory processes at 37°C.

Determination of soluble serum DPP-4 enzyme activity

The determination of serum DPP-4 enzyme activity was realized using the microplate-based kinetic method (Multiscan EX Labsystems) at 405 nm, 25°C in 30 minutes from duplicates. DPP-4 cleaves peptide bonds specifically after proline (and alanine) to a distance of two amino acids from the N-terminal end of the peptides. As a result of this, it cleaves off a para-nitroaniline molecule from the Gly-Pro-para-nitroaniline compound applied, which can be detected at 405 nm and whose quantity can be determined photometrically. Enzyme activity was determined from absorption values measured between 0. and 30. minutes at a temperature of 25°C. We determined enzyme activity in nmol/ml/min (U/L).

Determination of lymphocyte membrane-associated CD26 expression

The determination of the expression of CD26 bound to the surface of CD3+ and CD4+, CD8+ T lymphocytes was realized using the FACS (Fluorescence Activated Cell Sorting) method. We used anticoagulated full blood collected in full EDTA tubes as samples. We marked the cell surface markers by immunostaining. We determined CD26 positivity in MFI (mean fluorescence intensity).

Determination of serum ICA and GAD antibodies using ELISA kit

The determination of ICA and GAD antibodies was realized with the help of Medizym ICA and anti GAD ELISA kit (Medipan GmbH) from serum samples. We detected the samples using the spectrophotometric method at 450nm with a Multiscan EX ELISA reader. For the evaluation, we used Ascent software. We determined the results applying the standard curve obtained from the calibrator samples in the kit. From the values thus adjusted, we considered the samples with a concentration higher than 5 IU/ml positive and the ones with a concentration lower than 5 IU/ml negative.

Statistical methods

The determination of the statistical distribution of the results of the analysis was realized using the Jarque-Bera test. With a view to the fact that the results showed normal distribution, we applied the two-sample T-test and the Pearson correlation as well as ANOVA and MANOVA tests in order to analyse correlations and compare average values. We considered the P value inferior to 0,05 significant ($p<0,05$). For the establishment of the diagnostic cut-off point for the DPP-4 enzyme activity we applied the ROC (Receiver Operating Characteristic) curve analysis as a statistical examination.

Results

Determination of fasting and postprandial serum DPP-4 enzyme activity in type 1 and type 2 diabetic patients as well as in healthy individuals.

1. Determination of serum DPP-4 enzyme activity in diabetic patients in fasting state:

We measured significantly higher fasting serum DPP-4 enzyme activity in T1DM [29.065 U/L (95%CI:27.30-30.826)] in comparison to both healthy individuals [25.45 U/L (95%CI:24.16-26.76)] and the T2DM patient group [24.10 U/L (95%CI:22.75-25.46)] (T1DM vs. CNTRL p<0.0075; T1DM vs. T2DM p<0.0001).

2. Examination of serum DPP-4 activity variation in the case of carbohydrate intake:

We did not detect significant variation in serum DPP-4 enzyme activity following test food intake in either group (T1DM, T2DM, CNTRL):

T1DM: 0':29.07U/L, 95%CI:27.30-30.82U/L;
60':31.33U/L, 95%CI:25.83-36.82, n=15;
180':30.08U/L, 95%CI:25.57-34.50U/L, n=15;
T2DM: 0':24.10U/L, 95%CI:22.75-25.46U/L;
60':27.84U/L, 95%CI:24.6-30.9U/L, n=17;
180': 27.05U/L, 95%CI:22.82-31.27U/L, n=17;
CNTRL 0':25.45U/L, 95%CI:24.16-26.76U/L;
60': 24.42U/L, 95%CI:24.34-27.43U/L, n=18;
180': 25.86U/L, 95%CI:24.66-29.41U/L, n=18.

3. With respect to fasting serum DPP-4 enzyme activity and the clinical laboratory values related to the carbohydrate metabolism we did not find significant correlation in either group between fasting plasma glucose, or HbA1C and fasting DPP-4 enzyme activity values.

Examination of serum DPP-4 activity and lymphocyte membrane-bound CD26 expression in T1DM, focusing on the background of elevated serum DPP-4 activity

In addition to determining the fasting soluble serum DPP-4 activity, we also determined the expression of CD26 bound to the surface of the T-lymphocytes, which, at the same time, plays a role in T-cell activation and proliferation, in CD3+ lymphocyte and subsequently CD4+ and CD8+ lymphocyte subpopulations in T1DM on the one hand and healthy individuals on the other:

4. We detected significantly higher fasting serum DPP-4 activity in the T1DM group again (30,06U/L, 95%CI:21,85-45,94U/L)in comparison to the healthy group (22,62U/L, 95%CI: 16.32-28,28U/L) (ANOVA p=8.75e-12).

We measured significantly lower values in all lymphocyte populations examined regarding T lymphocyte membrane-bound CD26 expression in the T1DM group as compared to the healthy group.

- In **CD3+**lymphocytes: T1DM:100.32MFI;95%CI:92.91-107.72; CNTRL:119.82MFI, 95%CI 107.81-131.84MFI (ANOVA p=0.001154)
- In **CD4+** lymphocytes: T1DM:89.29MFI, 95%CI:83.45-95.13; CNTRL:106.48MFI, 95%CI 94.97-117.99MFI (ANOVA p=0.03294)
- In **CD8+** lymphocytes: T1DM:110.75MFI, 95%CI 98.42-123.08; CNTRL:136.45MF, 95%CI 121.22-151.68MFI (ANOVA p=0.00478)

MFI: mean fluorescent intensity

5. We did not find any correlation between serum DPP-4 activity and membrane-bound CD26 expression measured on the surface of lymphocytes in either lymphocyte subpopulation examined in T1DM.

CD3+ CD26: DPP-4: r²=0.0299; r=-0.1729; p=0.1052

CD4+ CD26: DPP-4: r²=0.177; r=-0.1329; p=0.3526

CD8+ CD26: DPP-4: r²=0.0042; r=-0,002; p=0.9891

Examination of autoimmune ICA and GADA activity in relation to soluble serum DPP-4 and lymphocyte surface-bound CD26 in T1DM patients.

6. Within the T1DM group, from among the 48 patients, isolated ICA positivity was detected in 14 cases, only GADA positivity in 2 cases, positivity for both autoimmune markers in 27 cases and negativity to both markers in 5 cases.

7. We did not find any correlation between serum DPP-4 activity and the presence of autoimmune markers regarding any of the autoimmune markers measured. Neither did we find any correlation between enzyme activity and the autoimmune markers in the patients that turned out to be negative for both autoimmune markers. However, independently of the presence or the absence of the autoantibody, serum DPP-4 activity was elevated.

8. We did not find any correlation between the presence of the autoimmune markers and lymphocyte surface CD26 expressions in either lymphocyte subpopulation.

With a view to the fact that we found considerable variance between the fasting DPP-4 activities of T1DM patients and the control groups, we carried out a ROC analysis in order to examine the diagnostic efficiency of DPP-4 activity to find out whether elevated DPP-4 activity can be considered as a marker of type 1 diabetes:

Serum DPP-4 activity as an independent diagnostic test in the diagnosis of type 1 diabetes:
Regarding DPP-4 activity, the cut-off value established via ROC analysis as an independent diagnostic test is 25.91 U/L. Using this cut-off value, the sensitivity of DPP-4 activity as an independent test is 76.6% and its specificity is 88%.

ICA and GAD autoantibodies as a combined diagnostic test in T1DM:

In the case of applying ICA and GADA markers as a combined diagnostic test, the sensitivity of the test is 89,4% (13.4 ± 9.76 years after the establishment of the diagnosis of T1DM).

ICA, GADA and serum DPP activity as a combined diagnostic test in T1DM:

In the case of applying ICA, GADA markers and serum DPP-4 activity as a combined diagnostic test, the sensitivity of the test is 95,7%.

Conclusions

Based on our examinations, the role of DPP-4 in immune processes can also be expected to be decisive in the pathomechanism of the autoimmune T1DM. Based on our findings, we came to the following conclusions:

1. We demonstrated that serum DPP-4 enzyme activity is always elevated in T1DM, independently of carbohydrate intake or the status of one's carbohydrate metabolism comparing to the T2DM or CNTRL group. This indicates that elevated serum DPP-4 enzyme activity does not vary depending on the metabolism status.
2. We demonstrated that in T1DM, membrane-bound CD26 expression is decreased on the surface of T-lymphocytes. This may be related to the autoimmune nature of the basic process.
3. We did not find any direct correlation between the carbohydrate metabolism and other important clinical parameters, the enzymatic activity of the DPP-4 serum examined on the one hand and its T-lymphocyte cell surface expression on the other in any of the groups studied. Thus it seems likely that the variances detected do not vary depending on changes in the carbohydrate metabolism.
4. We demonstrated that when applied as a combined diagnostic test, serum DPP-4 activity increases the sensitivity of the autoimmune markers examined in T1DM if the control group contains healthy individuals only; however, when applied independently, it is not appropriate for diagnosing T1DM due to its low specificity.

Taking into consideration in T1DM the elevated serum DPP-4 enzyme activity, the decreased CD26 expression on the surface of the T-lymphocytes, the autoimmune nature of the disease and the role of the protein in the immune processes, the background of the elevated enzyme activity is likely to include the following:

1. The autoimmune process itself, which may be further confirmed by the decreased CD26 expression detected on the surface of the T-lymphocytes. The decreased expression on the surface of the T-lymphocytes may theoretically be a sign of dysfunction as well because of the CD26 costimulation signal. The process may form part of the regulatory T-cell dysfunction not entirely unravelled so far in T1DM.
2. A hormonal feedback mechanism, in which an increased incretin (GLP-1, GIP) hormone secretion aimed at the maintenance and protection of the decreased beta-cell mass and insulin production may be followed by the consequently increased DPP-4 activity. In the current examination, we did not have the opportunity to measure incretins.
3. A possible target organ damage. (In the patients participating in the examination, we did not detect any correlation between the presence of diabetic retinopathy, microalbuminuria, or the development of neuropathy and the variation of soluble or membrane-bound DPP-4 expression.)
4. Theoretically, the presence of autoimmune concomitant diseases (RA, Basedow-Graves, SLE). However, we could not confirm this presumption in the present population selected and examined.
5. A previous viral infection (EBV) or chronic viral infection, e.g. hepatitis C also triggers higher serum DPP-4 enzymatic activity. The development of type 1 diabetes has been associated to several former viral infections (CMV, coxsackie, parvo, rota, rubella).

We were the first to describe that the serum DPP-4 activity belonging to the enterohormonal axis changes in T1DM. Higher serum enzyme activity may serve as a partial basis for possible later clinical examinations, in which DPP-4 inhibitors would be applied.

Based on our examinations, we came to the conclusion that both the variation of soluble DPP-4 and that of T-lymphocyte surface-bound CD26 expression are characteristic of T1DM. Our findings demonstrate that both the study of serum DPP-4 enzyme activity and that of T-lymphocyte surface CD26 expression may play a role in the regulation of the immune-regulatory processes of autoimmune T1DM, whose understanding urges further examinations.

Papers of the author in the scope of the present work

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3. Firneisz G, **Varga T**, Lengyel G, Fehér J, Ghyczy D, Wichmann B, Selmeci L, Tulassay Z, Rácz K, Somogyi A. (2010) Serum dipeptidyl peptidase-4 activity in insulin resistant patients with non-alcoholic fatty liver disease: a novel liver disease biomarker. *PLoS One* 5(8):e12226.