Original Article

Evaluation of the Enzymatic Activity of Soluble CD13/APN and CD26/DPP4 in Serum and Urine Samples of Mice with Experimental Autoimmune Encephalomyelitis

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Abstract

Background: Dipeptidyl Peptidase IV (DPP4/CD26) and Amino Peptidase N (APN/CD13) have essential roles in inflammatory diseases. The current study aimed to determine changes in APN and DPP4 enzyme activity in the serum and urine of mice with experimental autoimmune encephalomyelitis (EAE).

Materials and Methods: In the present study, female C57BL/6 mice were studied in two groups (control and EAE). Twenty-sevendays after induction, the enzymatic activity of APN and DPP4 in urine and serum samples was measured using a spectrophotometric assay.

Results: The enzyme activity of DPP4 was higher in serum and urine of EAE mice than in the control group (mean in serum: 1.04 ± 0.13 pmol/mL and 0.80 ± 0.12 pmol/mL, respectively, P=0.015; mean in urine: 0.26 ± 0.04 pmol/mL and 0.19 ± 0.04 pmol/mL, respectively, P=0.015). However, the enzymatic activity of APN in serum and urine of mice with EAE when compared to the control group had no significant difference (mean in serum: 9.20 ± 1.15 unit/mL and 10.25 ± 1.21 unit/mL, respectively, P=0.132, mean in urine: 0.23 ± 0.27 unit/mL and 0.15 ± 0.05 unit/mL, respectively, P=0.310).

Conclusion: The increased DPP4 activity along with normal APN activity in urine and serum samples can be used as an indicator to detect or follow up on the course of MS disease. Confirmation of this finding needs further investigation.

Keywords: Aminopeptidase N, CD13, CD26, Dipeptidyl peptidase 4, Experimental autoimmune encephalomyelitis

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Introduction

Multiple Sclerosis (MS) is a chronic autoimmune disease of the central nervous system (CNS) that affects more than 2 million people worldwide[1]. Multiple Sclerosis is mainly characterized by progressive demyelination and localized inflammation in the brain and spinal cord[2]. While the leading cause of

MS is unknown, animal models and patient studies have shown that abnormal activity of various types of lymphocytes against myelin proteins plays a vital role in the pathogenesis of the disease[3].

There is no single diagnostic test for MS; diagnostic criteria include both clinical and magnetic resonance imaging (MRI) findings that rely on the absence of any alternative diagnosis. Therefore, remains a need for biomarkers that can be used in both the diagnosis and monitoring of MS progression.

It has previously been shown that aminopeptidase N (APN; CD13, E.C. 3.4.11.2) and dipeptidyl peptidase IV (DPP4, CD26, EC 3.4.14.5) activity are associated with the inflammatory responses in MS[4,5], APN is a zinc-dependent metallopeptidase of the superfamily of gluzincins[6] and DPP4 is a 110-kDa transmembrane multifunctional serine protease[7]. APN is involved in cell migration, extracellular matrix degradation, and antigen processing[5] by hydrolyzing a broad spectrum of biologically active peptide substrates. APN stops hydrolysis if proline is in the second position of the N-terminal sequence, thus generating potentially DPP4-susceptible substrates[8]. Likewise, DPP4 cleaves many biologically important substrates, including several neuropeptides and chemokines[9]. DPP4 mainly targets glucagon-like peptide, which regulates glucose metabolism and is mainly responsible for reducing glucose concentrations in the context of type 2 diabetes mellitus (T2DM).

APN and DPP4 have a widespread distribution, occurring on epithelial cells, fibroblasts, endothelial cells, and kidney[10]. Moreover, APN is expressed by monocytes/macrophages, granulocytes, and also activated T cells. On the other hand, DPP4 constitutively is expressed on lymphocytes. Indeed, T helper 17 cells that have a pivotal role in MS pathology show the highest expression of DPP4[11]. APN and DPP4 also exist in soluble form (sAPN and sDPP4, respectively), and they are present in different biological fluids such as plasma where at least 95% of their enzymatic activity is found [12], seminal fluid and in low amounts in the cerebrospinal fluid (CSF)[13].

Regarding the pivotal roles of these ectoenzymes, it is presumed that the development of inflammatory and autoimmune diseases is correlated with the disturbances of these molecules' regular activity or expression. Hafler *et al.*[14] published the first study that showed that the frequency of CD26+ T cells increased in the peripheral blood and the CSF of MS patients with progressive forms of the disease. Others found that CD26 expression decreases somewhat in peripheral blood and cerebrospinal fluid after treatment[15]. However, Subsequent studies presented conflicting results[16], but it was eventually confirmed that circulating CD26+ T cells correlate with clinical and MRI disease activity scores in MS[17,18]. Of note, the expression of CD13/APN was also found to be increased on PBMC in patients with MS[19]. In recent studies, Ziaber *et al.* presented evidence for APN playing a role as an immunological marker of various clinical forms of MS.

Moreover, it has been shown that simultaneous inhibition of DPP4 and APN enhances TGF-β production and down-regulates T cell functions in the central nervous system[20]. Based on the results of these studies, DPP4 and APN enzymatic activity has attracted significant interest as a biomarker and a potential treatment target of MS[21]. Soluble DPP4 and APN are considered prognostic criteria for several inflammatory and autoimmune diseases, including MS (PMID: 28448874). Prior studies in MS patients or experimental autoimmune encephalomyelitis (EAE) animal models have focused on the sDPP4 and sAPN activity on plasma or CSF. However, the potential presence of MS/EAE biomarkers in urine is largely neglected. This study aimed to determine plasma and urinary activity levels of APN and DPP4 as potential biomarkers in EAE.

Methods

EAE Induction and Histological evaluation of spinal cord tissue: Twelve female C57BL/6 mice (6-8 weeks) were obtained from the Royan Research Center of Tehran and randomly divided into EAE and control groups. Mice were housed and treated according to the ethical protocols for animal treatment and handling Shahid Beheshti University of Medical Sciences (Ethical approval code: IR.SBMU.MSP.REC.1396.454).

To induce EAE, mice were anesthetized by peritoneal injection of the combination of 80 µL ketamine hydrochloride and 20µL xylazine. 200 µg of the MOG_{35-55} peptide (MOG35–55; KJ Ross-Petersen ApS, Copenhagen, Denmark) mixed with the same volume

of complete Freund's adjuvant (CFA; Sigma, F5881, USA) containing 500 µg of killed Mycobacterium tuberculosis (final volume of 100 µL). The injections were done subcutaneously in both hind flanks of mice. Thirty minutes later, mice have received pertussis toxin (300 ng in 100 µL PBS; List Biological Lab, Campbell, CA, USA) intraperitoneally. The injection of pertussis toxin was repeated 48h later.

The clinical symptoms were evaluated every other day on a scale from 0 to 7 according to the following criteria: 0 = no symptoms; 1 = distal limp tail; 2 = complete limp tail; 3 = one hind limb paralyzed; 4 = both hind limbs paralyzed; 5 = Hind limbs and one forelimb paralyzed; 6 = Hind limbs and both forelimbs paralyzed; 7 = moribund/death[22]. EAE mice were sacrificed 27 days after induction of the disease, and spinal cords were removed for histological analysis. Infiltration of inflammatory cells and axonal destruction are then investigated using standard Hematoxylin and Eosin (H&E) and Luxol Fast Blue (LFB) staining, respectively[23].

Serum and urine Sample Collection: Urine and serum samples of all studied animals were collected 27 days after induction of the disease. One mL of blood sample was collected through retro-orbital sinuses. All samples were stored at −20°C for further use.

Measurement of CD13/APN and CD26/DPPV Enzymatic Activity by Spectrophotometric Method: The serum and urine samples were collected at the peak of the disease on the 27th day after disease induction. The enzymatic activity of CD13/APN was measured using a modified method by Behzadi *et al.*[24] Briefly, 90 µl of substrate mixture [1 mM L-leucine p-nitro aniline (SIGMA) in methanol] and 10 µl of serum were used. Release of p-nitro aniline from L-leucine p-nitro aniline was measured at a wavelength of 405 nm after 60 min using a microplate reader (Anthos 2020 UK-Biochrom). The mM extinction coefficient of p-nitro aniline at 405 nm is 10.8 nm, so APN enzyme activity was calculated using the following formula:

$$\text{Unit/ml} = \frac{(\text{A405nm test} - \text{A405nm Blank}) \times (\text{Dilution factor})}{(10.8)(0.1)}.$$

DPP4 activity was measured using one mM chromogenic substrate Gly-Pro-Gly-Pro-p-hydrochloride (pNA, Sigma) in 100 mL Tris-HCl pH 8 buffer[25]. 10 µL of serum was diluted with 90 µL of 100 mM Tris-HCl buffer, and then 100 µL of the prepared substrate was added to each well and incubated at 37° for 15 min. The reaction was stopped by the addition of 1 M sodium acetate buffer at pH 4.4. after centrifugation for 5 minutes (10,000 × g), the hydrolyzed substrate was measured by optical absorption at 405 nm (p-nitro anilide production) using an Anthos 2020 microplate reader (Emax, Molecular Devices). Negative control of reaction was prepared by adding sodium acetate buffer before adding the substrate. To determine the concentration of released p-nitro aniline, a standard curve of p-nitro aniline was prepared[26]. All experiments were repeated three times.

Statistical analysis: Differences in the mean enzymatic activity of APN and DPP4 in the EAE and control groups were analyzed by the Mann-Whitney U test, and P values ≤ 0.05 were considered statistically significant. Data were analyzed by SPSS software (version 21). Graph-Pad Prism (GraphPad Software, Inc., San Diego, CA version; 6) was used to generate the graphs.

Results

Clinical symptoms and Histopathological evaluation: Experimental autoimmune encephalomyelitis was induced in C57BL/6 mice via immunizing them using an emulsion of MOG mixed with CFA. Mice showed the first symptoms of the disease at 12 ± 1 days after immunization, and the peak of the disease was observed 20±3 days after disease onset (Figure 1A). s the disease progressed, the EAE mice loosed their weight (Figure 1B). Twenty-seven days after immunization, mice were sacrificed, and histological analysis was performed. LFB staining together with H&E staining in the spinal cord of EAE mice revealed pathology, including large foci of degenerating myelin and infiltration of inflammatory cells, mostly in the caudal regions of the brain spinal cords. The utmost conspicuous changes were observed in mice showing the most severe symptoms (clinical score 2.0 or greater). (Figure 1C & D).

Measurement of APN and DPP4 activity: Spectrophotometric methods were used to measure the activity of APN and DPP4 in serum and urine of the EAE and control groups. The urine and serum samples

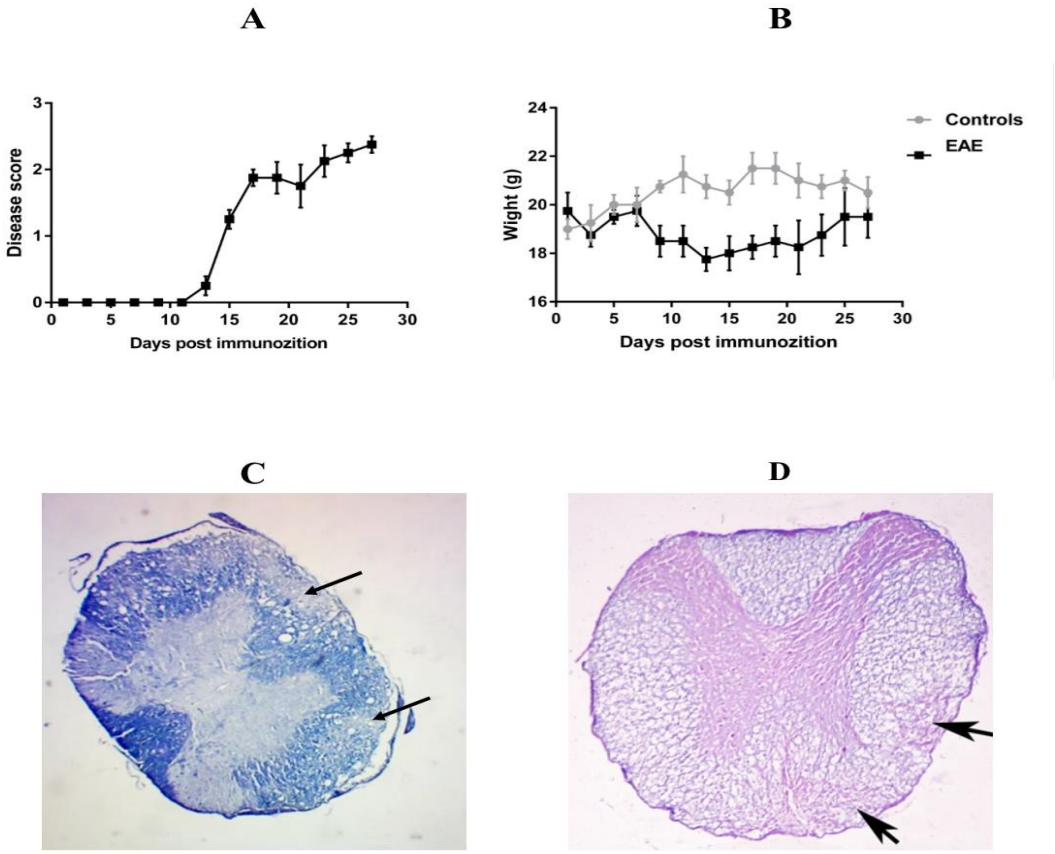

Figure 1. Clinical signs and body weights of Experimental autoimmune encephalomyelitis mice. (A) Clinical signs were monitored every other day by two examiners. EAE signs emerged on the 12th-day post-induction of EAE. (B) The weight of mice was measured every other day. The data are the mean ± standard error of the mean (SEM) of six animals per group. (C) Luxol fast blue staining was used to evaluate demyelination in spinal cords. Arrows indicate areas of demyelination in one representative image. (D) Haemotoxylin and Eosin staining revealed the area in spinal cords that of inflammatory cells infiltrated (arrows depict the area).

were collected on the 27th-day post-immunization. In healthy and EAE mice, the activity of both enzymes in the urine samples was lower than their activity in sera.

The level of DPP4 enzyme activity was higher in the serum and urine of mice with EAE than in the control group (Figure 2A and B). Results showed that the mean ± standard deviation (SD) of DPP4 in serum of EAE mice was significantly higher than controls (1.04 ± 0.13 pmol/mL and 0.80 ± 0.12 pmol/mL, respectively, P = 0.015). Urine analysis revealed that DPP4 activity was higher in EAE mice when compared to controls (0.26 ± 0.04 pmol/mL and 0.19 ± 0.04 pmol/mL, respectively, P=0.015). However, no changes in serum and urinary APN enzyme activity were observed in EAE mice when compared with health mice (Figure 2C and D). The mean ± SD of serum APN activity in EAE and the control mice was 9.20 ± 1.15 unit/mL and 10.25 ± 1.21 unit/mL, respectively, (P= 0.132). Similarly, urinary activity of APN in EAE and controls was 0.23 ± 0.27 unit/mL and 0.15 ± 0.05 unit/mL, respectively (P = 0.310).

Discussion

Most brain disease biomarker studies have focused on analyzing CSF and blood samples[27]. On the other hand, most studies on urinary biomarkers have focused on kidney diseases due to the close relationship between the kidneys and urine[28]. The lack of attention to urinary biomarkers in other diseases, like brain diseases, may be because anatomically, the brain and urine are not closely related, and also there is the filtering effect of the blood-brain barrier and the kidneys. Nevertheless, it could be assumed that changes in organs such as the

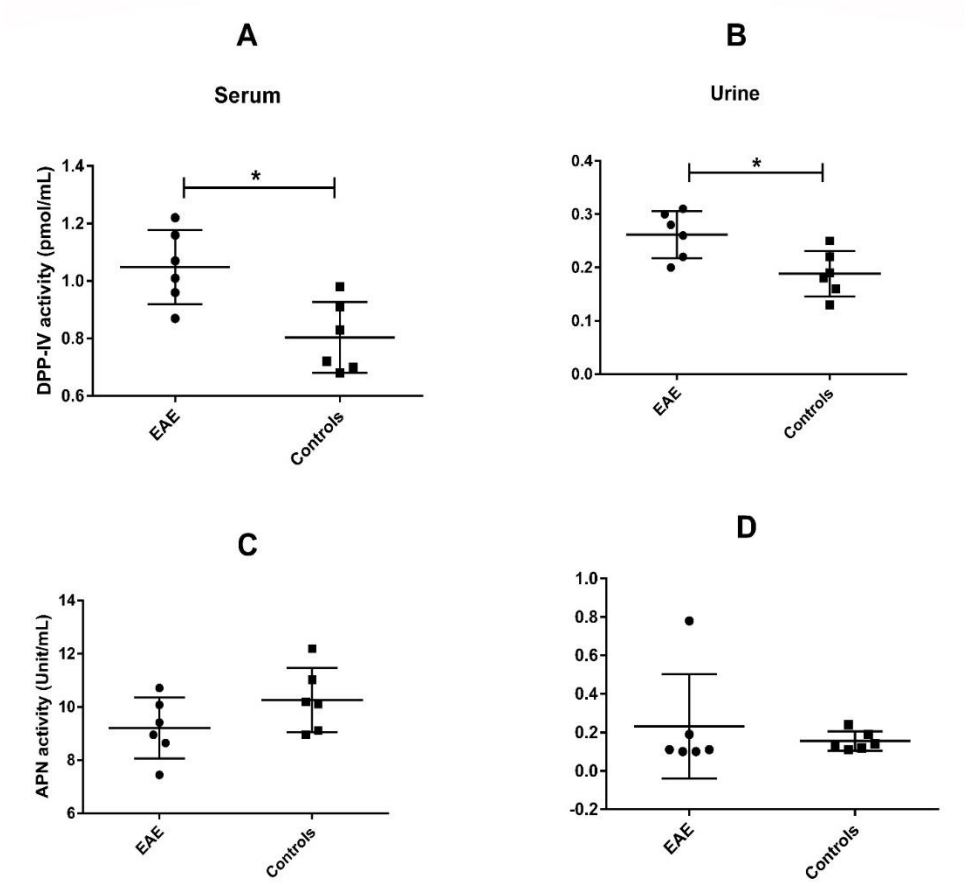

Figure 2. Measurement of DPP4 and APN6 activity in serum and urine samples. Serum and urine samples were collected on the 27th-day post disease induction. DPP4 and APN activity was measured using spectrophotometric methods. Mann-Whitney U test was used to analyze differences between groups. P values less than 0.05 were considered significant. The data are expressed as the mean ± standard error of the mean (SEM) of six mice per group.

brain and immune system are somehow reflected in the urine. Unlike blood, urine is not subject to homeostatic mechanisms. Therefore, more significant fluctuations could occur in urine than in blood, better reflecting the changes in the human body[29].

Regarding the fact that APN and DPP4 levels and the enzyme activity of them increase by inflammatory responses in MS/EAE, we examined the activity of these two enzymes in urine samples of EAE mice. The results of the curresnt study indicated that DPP4 enzyme activity in serum and urine of EAE mice is higher than in the control group, but there is no significant difference in APN enzyme activity in serum and urine samples of EAE and healthy mice.

Indeed, enzymatic activity and soluble level of DPP4 in serum are used as a marker in various chronic inflammatory diseases, including diabetes, tumors, hematological malignancies, immunological, inflammatory, psychoneuroendocrine disorders, viral infections, coronary artery disease, cancer,[30] and asthma[31]. Additionally, in MS, the elevation of DPP4 may arise from an increase in the frequency of activated T cells in patients, thus reflecting the presence of pathogenic myelin-reactive T cells. Previous studies showed that the median percentage of CD26+ cells among CD4+ T cells and CD8+ T cell populations in the peripheral blood of patients with MS were increased[18]. Others found correlations between changes in the frequency of CD26-expressing T cells and lesion activity in MRI of MS patients with relapsing-remitting and chronic progressive disease courses, consistent with the function of CD26 in regulating T cell activation states[17,18]. Jensen *et al.*[32] demonstrated that the percentage of blood CD26+ CD4+ T cells was

increased in the initial attack of MS, and correlated with disease activity measured by MRI and with clinical disease severity. Finally, gene expression profiling in MS patients and healthy controls identified higher average CD26/DPP4 in peripheral blood mononuclear cells of MS patients[33]. Collectively, these data indicate that increased CD26 expression and enzymatic activity on human CD4+ T cells correlates with disease activity in MS.

Soluble Aminopeptidase N (CD13) is a diagnostic biomarker for some malignant[34,35] and inflammatory diseases[36]. Additionally, increased expression of APN on peripheral blood mononuclear cells in patients with MS was reported previously[37]. However, we did not observe significant differences in APN activity in the urine and serum of healthy and EAE mice. Since renal dysfunction results in a simultaneous increase in APN and DPP4 activity[38], DPP4 activity and no change in APN activity can help differentiate kidney disease from EAE/MS.

Conclusion

The current study provides evidence that urine may be a good source for searching biomarkers of EAE; though the current results should be verified by subsequent large-scale clinical studies.

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