Characterization of human serum dipeptidyl peptidase IV (CD26) and analysis of its autoantibodies in patients with rheumatoid arthritis and other autoimmune diseases

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Abstract

Objectives

To assess the serum levels, specific activity and other characteristics of dipeptidylpeptidase IV (DPP IV/CD26), an ectoenzyme that plays a critical role in the modulation and expression of autoimmune and inflammatory diseases, from patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), primary Sjögren syndrome (SS) and normal controls. To study the possible underlying molecular basis if significant differences were found.

Methods

Serum DPP IV was purified by ion-exchange and affinity chromatography techniques and its specific activity and sera levels were determined by an enzyme-linked assay (ELISA). The enzyme was further analyzed for its sialic acid content, its adenosine deaminase binding capacity and its electrophoretic mobility. The levels of circulating IgA, IgG, and IgM anti-DPP IV autoantibodies were determined by an ELISA technique.

Results

The median serum levels of DPP IV in RA patients was similar to controls (0.85 g/ml versus 1.03 g/ml, p = n.s.); in SLE and SS patients the enzyme serum levels were reduced to nearly one half of controls (p < 0.001). DPP IV specific activity was significantly reduced in sera from RA patients when compared with those of SLE, SS and normal sera (12.24 versus 16.5, 19.69 and 16.34 mol pNA x 10^-4/min/mol respectively, p < 0.005). Both RA and SLE enzymes were hypersialylated, but only RA DPP IV augmented its specific activity to close to control values after desialylation with V. cholerae neuraminidase. Sera from all patient groups contained anti-DPP IV autoantibodies, but only those of the IgA isotype were significantly higher than those found in normal subjects.

Conclusion

The specific activity of serum DPP IV was decreased only in RA patients, although its levels were similar to normal controls. While both RA and SLE DPP IV were hypersialylated, desialylation restored the specific activity only of RA DPP IV. This finding suggests that different specific glycosylation sites in the enzyme might be involved as the underlying mechanism of the decreased enzyme specific activity of RA patients. The differences in DPP IV levels observed between RA and SLE patients seem to reflect a different status of T cell activation in both diseases.

Key words

Dipeptidyl peptidase IV, autoimmunity, rheumatoid arthritis, systemic lupus erythematosus, Sjögren’s syndrome.

**Introduction**

Dipeptidyl peptidase IV (DPP IV/CD26) is a widely distributed, multifunctional, highly glycosylated membrane-bound ectoenzyme (1) that cleaves X-Pro dipeptides from the NH2-terminus of a large variety of proteins (2). Expression of DPP IV is closely associated with cell differentiation and activation, and it is involved in T lymphocyte activation and migration across the extracellular matrix (3, 4). A soluble form of DPP IV is also found in human serum (5). Analyses of serum DPP IV activity show a correlation with the activity of the membrane bound enzyme on normal T lymphocytes (6). Serum DPP IV isoforms are not only analogous to the isoforms found in T lymphocytes (7), but they bind adenosine deaminase with similar specificity and affinity (8-10), suggesting that the serum enzyme originates from T lymphocytes. It has been recently shown that at least 95% of the serum DPP activity can be attributed to a protein with ADA-binding properties and structural characteristics identical to those of DPP IV/CD26 (11).

DPP IV participates in the degradation of IL1, IL2 and TNF as well as in the inactivation of eotaxin and many other proinflammatory molecules (12, 13). Thus DPPIV has a central role in the anti-inflammatory response.

Due to the key role that DPP IV plays in T cell-mediated immune responses and lymphokine synthesis (10), the enzyme has been studied in several autoimmune disorders such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). In patients with RA and SLE there is a reduction in serum DPP IV activity (14-18). This reduced DPP IV activity may contribute to generate a pro-inflammatory state in these diseases. However, the precise mechanism involved in this finding has not been clarified.

In the present study we assessed whether the reduced activity of serum DPP IV observed in patients with RA and SLE is due to either lower serum levels, or structural alterations of the molecule and/or to the action of anti-DPP IV autoantibodies. The enzyme was purified from the sera of patients with RA, SLE and SS and compared with that of normal controls. We studied the enzymatic activity, the degree of glycosylation and the presence of DPP IV autoantibodies in all four groups of patients.

**Patients and methods**

**Patients**

The protocol was approved by the Ethics Committees of both participating centers. After informed consent was obtained, serum samples were drawn from patients that were diagnosed and treated in the Clinic of Rheumatology, Veterans Affairs Medical Center, Durham, NC. Twenty-five patients with active RA (15 women and 10 men) fulfilling the American College of Rheumatology revised criteria for the classification of RA were studied (19). The RA patients had a mean age of 54 years and a disease duration of 7 years. Sera from 10 patients with active SLE (6 women and 4 men), fulfilling the ACR revised criteria for the classification of SLE (20), with a mean age of 45 years were also obtained. Sera from 10 patients suffering from primary Sjögren’s syndrome (8 women and 2 men), with a mean age of 52 years were also obtained from the same clinic (21). The sera from an additional group of 25 healthy persons (15 women and 10 men), paired by sex and age, were selected as controls.

**Proteins**

DPP IV from human serum was isolated by sequential purification using DEAE-Sepharose ion exchange chromatography, Gly-Leu affinity chromatography and gel filtration over Sephacryl S-200 as described by Shibuya-Saruta et al. (9), followed by affinity chromatography on immobilized adenosine deaminase (ADA) as described by De Meester et al. (22) with an overall yield of 60% over serum. Calf intestinal mucosa ADA was obtained from Sigma Chemical Co., St. Louis, MA.

**Enzyme assays**

DPP IV activity was measured in 96-well culture plates using Gly-Pro-p-nitroanilide (0.2 mM) as substrate in...
reaction mixtures (200 l) containing serum samples (40 l) and 50 mM Tris-HCl, pH 8.0. The hydrolysis of the substrate was monitored at a wavelength of 405 nm using an Anthos Labtec kinetic plate reader. Activity was expressed as A_{405} nm/min. Specific activity was calculated using a molar extinction coefficient of 8800 M^{-1} cm^{-1} for p-nitroanilide at 405 nm (23) and a molecular mass of 220,000 Da for DPP IV. All experiments were done in triplicate unless otherwise specified.

**Antibodies**

Antibodies to purified DPP IV from human serum were prepared in rabbits according to standard protocols (24). The polyclonal anti-DPP IV IgG was purified by affinity chromatography on Protein A-Sepharose (25) followed by immunoabsorption to DPP IV coupled to Sepharose 4B. The monoclonal anti-DPP IV IgG, IOA26-clone BA5, was purchased from AMAC, Inc. (Westbrook, ME). Human IgG Fc fragment and goat affinity purified F(ab')₂ fragments against human IgA, IgG and IgM were purchased from ICN Pharmaceuticals, Inc. (Aurora, OH). Secondary antibodies to human IgA, IgG and IgM were purchased from Sigma Chemicals Co. St. Louis, MO. Human anti-DPP IV IgA was purified from patient sera by chromatography on Jacalin-Sepharose (26) followed by immunoabsorption to DPP IV coupled to Sepharose 4B. Human anti-DPP IV IgG was purified from patient sera by chromatography on Protein A-Sepharose (25) followed by immunoabsorption to DPP IV coupled to Sepharose 4B. Human anti-DPP IV IgM was purified from patient sera by ion-exchange chromatography on MBP-Sepharose (27), followed by immunoabsorption to DPP IV coupled to Sepharose 4B.

**Gel electrophoresis**

Electrophoresis was performed on polyacrylamide gels (1.2 mm thick; 14 x 10 cm) containing 0.1% SDS. A discontinuous Laemmli buffer system was used (28). The dye-conjugated Mr markers (Bio-Rad, Richmond, CA, USA) used were: myosin (Mₙ = 218,000), α-galactosidase (Mₙ = 84,000), and carbonic anhydrase (Mₙ = 46,000). Visualization of the proteins was carried out by staining the gel with 0.25% Coomassie Brilliant Blue R-250 in 45% (v/v) methanol/10% (v/v) acetic acid.

**Sialic acid determination**

Sialic acid content was measured using the Glyko System (Glyko, Novato, CA) according to the manufacturer's instructions. Briefly, 10 g of glycoprotein was hydrolyzed, and the N released sialic acid was fluorescently labeled and electrophoresed on proprietary gels. Quantification of sugar was performed using a fluorescence imaging system connected to an IBM computer.

**Desialylation of DPP IV**

V. cholerae neuraminidase was incubated with 1 mM 3,4-dichloroisocoumarin and 1 mM o-phenanthroline for 30 min at room temperature and filtered through PTI-Sepharose to eliminate any contaminating proteolytic activity. Asialo-DPP IV was prepared by incubating DPP IV (5 g/ml) in 0.1 M sodium acetate, 2 mM CaCl₂, pH 5.4, containing 20 units of V. cholerae neuraminidase for 10 h at 37°C. The solution was then dialyzed overnight against 20 mM Hepes, pH 7.4, and DPP IV was repurified by chromatography on ADA-Sepharose (22).

**Enzyme-linked immunosorbent assays**

Enzyme-linked immunosorbent assays (ELISA) were performed in 96-well culture plates. For quantification of DPP IV, plates were coated first with 200 l of a solution containing 5 g/ml of an anti-DPP IV mAb (IOA26-clone BA5) in 0.1 M Na₂CO₃, and incubated overnight at 4°C. Non-specific sites were blocked by incubating with PBS-Tween containing 2% bovine serum albumin (PBS-Tween-BSA) at room temperature for 1 h. For assays, increasing concentrations of sera were added in triplicate in a 200 l final volume of PBS-Tween-BSA and incubated at 37°C for 2 h, followed by rinsing with PBS-Tween and incubation with an affinity purified polyclonal anti-DPP IV rabbit IgG (100 ng/well) at 37°C for 1 h. Plates were then washed with PBS-Tween and 200 l (1 alkaline phosphatase substrate (1 mg/ml p-nitrophenylphosphate) in 0.1 M glycine, 1 mM MgCl₂, 1 mM ZnCl₂, pH 10.4, was added to the plate, and absorbance was monitored at 405 nm using an Anthos Labtec Kinetic Plate reader. Bound DPP IV was expressed as A_{405} nm/min. Concentrations were calculated from a calibration curve constructed with affinity purified DPP IV. The concentration of anti DPP IV IgA, IgG and IgM in sera of patients was determined by ELISA using 96-well culture plates coated with DPP IV as previously described.

ADA binding assays were carried out by an ELISA technique in 96-well culture plates using calf intestinal enzyme as described by DeMeester et al. (22). Plates were coated first with ADA (5 g/ml) as described above. The plates were incubated with increasing concentrations of purified DPP IV for 2 h at 37°C and then washed three times with PBS-Tween and incubated with an affinity purified anti-DPP IV rabbit IgG (100 ng/well) for 1 h at 37°C. Plates were then washed with PBS-Tween and incubated with an alkaline phosphatase-conjugated Protein A for 30 min. Bound DPP IV was measured as described above.

**Statistics**

Due to the relatively small sample size and some deviation from the normal distribution of some study variables, the main statistical analysis was performed by means of non-parametric tests. The statistical significance of differences between the variables DPP IV levels, DPP IV specific activity and IgG, IgA and IgM antibodies against DPP IV was evaluated by means of Kruskal-Wallis ANOVA. The specific post hoc comparisons within each group of variables was performed by means of the Mann-Whitney U test.

**Results**

DPP IV levels in sera of patients with RA and autoimmune disorders

The median serum levels of DPP IV in normal controls was 1.03 g/ml. Among patients it was 0.85 g/ml for RA, 0.42 g/ml for SLE and 0.45 g/ml for CD26 autoantibodies in patients with autoimmune diseases / M. Cuchacovich et al.
g/ml for SS (Fig. 1). The overall difference between groups was statistically significant (p < 0.0001), but this difference was due to the similarly decreased levels of DPP IV in SLE and SS patients (nearly one half the values seen in the normal controls). No statistically significant difference was found in DPP IV sera levels in RA patients versus normal controls (p > 0.705).

**DPP IV specific activity in sera of patients with RA and autoimmune disorders**

The median of the individually calculated overall specific DPP IV activity in the sera of the four groups were: 12.24 mol pNA x 10^{-4}/min/mol for RA, 16.5 mol pNA x 10^{-4}/min/mol for SLE, 19.69 mol pNA x 10^{-4}/min/mol for SS, and 16.34 mol pNA x 10^{-4}/min/mol for normal controls (Fig. 2). The overall difference among groups was statistically significant (p < 0.02), due to the decrease in DPP IV activity in RA patients. The differences in DPP IV specific activity in the sera of SLE and SS patients compared to normal controls were not statistically significant (p > 0.51).

**Characterization of DPP IV in sera of patients with autoimmune disorders**

Figure 3 shows protein bands in the Mr ~116,000 range, corresponding to a monomer of DPP IV. Sialic acid analyses (Table I) show that serum DPP IV of normal controls and SS patients have a similar sialic acid content (mean 4.69 and 4.58 pmol/pmol protein, respectively). However, RA and SLE DPP IV have a higher content of sialic acid (means 10.71 and 12.08 pmol/pmol protein, respectively; p < 0.002). Comparison of specific activities among the purified enzymes standardized by protein concentration (Table II) shows that only RA DPP IV specific activity was decreased when compared with its normal counterpart (mean 6.10 and 16.15 mol pNA x 10^{-4}/min/mol DPP IV, respectively). When the enzymes were desialylated with *V. cholerae* neuraminidase, only the specific activity of RA DPP IV was augmented (Table II), whereas the SLE enzyme activity remained unchanged. These findings suggest that the lower activity observed in serum DPP IV from RA patients is caused by hypersialylation.

**Adenosine deaminase binding assays of DPP IV from patients with autoimmune disorders**

Figure 4 shows that the ADA-binding abilities of RA and SS serum DPP IV do not differ significantly from that of normal DPP IV; however, the SLE enzyme shows an augmented binding ability to ADA, suggesting that hypersialylation of DPP IV in SLE patients may enhance its reactivity with ADA.

**Anti-DPP IV antibodies levels among RA, SLE, SS patients and normal controls**

The median of the serum levels of anti DPP IV IgA autoantibodies was 9.02...
Table I. Sialic acid content of DPP IV isolated from sera of patients with autoimmune disorders.

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<tr>
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<th>Sialic acid (pmol/pmol protein)</th>
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<tr>
<td>Normal</td>
<td>4.69 ± 0.47</td>
</tr>
<tr>
<td>RA</td>
<td>10.71 ± 0.68</td>
</tr>
<tr>
<td>SS</td>
<td>4.58 ± 0.60</td>
</tr>
<tr>
<td>SLE</td>
<td>12.08 ± 0.73</td>
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Table II. Effect of desialylation on specific activities of purified DPP IV isolated from sera of patients with autoimmune disorders*.

<table>
<thead>
<tr>
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<th>Specific activity (mol pNAx10^-4/min/mol DPP IV)</th>
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<tr>
<td>Normal</td>
<td>16.16 (± 1.55) x 10^-4</td>
</tr>
<tr>
<td>Normal + neuraminidase</td>
<td>15.63 (± 1.83) x 10^-4</td>
</tr>
<tr>
<td>RA</td>
<td>6.10 (± 0.45) x 10^-4</td>
</tr>
<tr>
<td>RA + neuraminidase</td>
<td>10.43 (± 2.08) x 10^-4</td>
</tr>
<tr>
<td>SS</td>
<td>11.50 (± 0.80) x 10^-4</td>
</tr>
<tr>
<td>SS + neuraminidase</td>
<td>10.23 (± 1.27) x 10^-4</td>
</tr>
<tr>
<td>SLE</td>
<td>11.40 (± 1.07) x 10^-4</td>
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<tr>
<td>SLE + neuraminidase</td>
<td>11.83 (± 1.53) x 10^-4</td>
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*DPP IV samples (5 g) were incubated with V. cholerae neuraminidase at 37°C for 10 h. Activity was determined in triplicate as described in the Methods section.

Fig. 4. ADA binding assay of serum DPP IV from patients with autoimmune disorders and normal controls. ( ) normal DPP IV, ( ) RA DPP IV, ( ) SS DPP IV, ( ) SLE DPP IV.

Fig. 5. Anti-DPP IV antibodies titers among normal controls (n=25), RA patients (n=25), SLE patients (n=10) and SS patients (n=10). A = anti-DPP IV IgA titers, B = anti-DPP IV IgG titers, C = anti-DPP IV IgM titers.

IgG antibody levels in RA patients was 1.64 g/ml, in SLE patients it was 1.57 g/ml, in SS patients it was 2.31 g/ml, and in normal controls it was 1.63 g/ml (Fig. 5B). Statistically, there were no overall differences in IgG anti-DPP IV autoantibody levels between groups (p < 0.49).
except for a slight increase in SS patients versus controls (p < 0.02). Similarly, serum levels of anti-DPP IV IgM autoantibodies were 44.31 g/ml in RA, 38.78 g/ml in SLE, 50.60 g/ml in SS patients and 38.69 g/ml in normal controls, respectively (Fig. 5C). There were no statistically significative differences between patient groups nor between patients and normal controls. Anti-DPP IV IgG antibodies of the IgA, IgG and IgM classes were purified from the serum of patients showing high titers and mixed with purified DPP IV to study their effect on the activity of the enzyme. Although they show specific binding to DPP IV by a Western blot technique, their effects were similar to those presented in Table II for untreated enzymes, suggesting that these autoantibodies do not affect the activity of DPP IV (data not shown).

Discussion
The physiological role of DPP IV in the expression or modulation of autoimmune and inflammatory diseases has been the subject of several studies. Two early studies demonstrated that DPP IV activity was decreased in the sera of RA and SLE patients (17,18), whereas a recent report (30) suggested that the levels of activity of DPP IV in RA and control plasma samples were not significantly different. Because no correlation was found between the peripheral lymphocyte count and serum DPP IV activity in SLE patients (18), we focused our study on the serum levels and molecular characteristics of soluble DPP IV. We found that DPP IV serum levels in RA patients were similar to those of normal controls; however, they were decreased in the sera of SLE and SS patients when compared with normal controls. While the concentration of DPP IV in the sera of RA patients appeared normal, its specific activity was significantly reduced.

DPP IV purified from human serum lacks the transmembrane domain of the membrane-bound enzyme (31); however, its enzymatic activity or ADA binding capacity is not impaired (31). Another form of DPP IV, which is not a cleavage product of DPP IV/CD26, is also found in human serum (32). This protein exists as a monomer of 175 kDa and its N-terminal sequence has no homology with DPP IV/CD26 (32), resembling more the activated T-cell antigen DPPT-L which does not bind to ADA (33). Using a combination of chromatographic and affinity techniques we purified a DPP IV protein resembling the enzyme described by Iwaki-Egawa et al. (30).

We found that the sialic acid contents of the RA and SLE enzymes were almost twice those found in the proteins from SS patients and normal controls. However, only the activity of RA DPP IV was affected as a consequence of hypersialylation. A similar decrease of DPP IV activity due to hypersialylation has been observed in the enzyme from peripheral blood T cells of AIDS patients (34). There are eight possible Asn-glycosylation sites in DPP IV (35), but modifications in only one of these sites – Asn319 – have the potential to modify DPP IV enzyme activity (35). Our findings suggest that DPP IV in RA and SLE patients may be hypersialylated at different Asn-glycosylation sites, since treatment of the enzyme with V. cholerae neuraminidase increased only the specific activity of RA DPP IV. Furthermore, the ADA binding capacity of SLE patients’ DPP IV appears to be augmented, whereas its activity is not affected by hypersialylation.

We also investigated whether anti-DPP IV antibodies may modify enzyme activity. Our results show that sera levels of anti-DPP IV autoantibodies of the IgA class were significantly higher in the sera of RA, SLE and SS patients when compared with those of normal controls. Serum levels of anti-DPP IV autoantibodies of the IgG and IgM classes in the three groups of patients were not significantly different from those of normal controls, suggesting that only anti-DPP IV autoantibodies of the IgA class may have some relevance in the immune response against DPP IV. Although antibodies produced in mice and rabbits downregulate some of the functions of DPP IV (36), isolated anti-DPP IV autoantibodies do not modify substantially the activity of the enzyme.

Newly synthesized DPP IV is cleared from the circulation by the liver basolateral endosomes in association with polymeric IgA receptors (pIgA-R), responsible for the transportation of IgA to bile, via a joint transcytotic process (37). This process has been also suggested as a major mechanism for the clearance of antigens from the circulation in the form of IgA-antigen complexes via bile secretion (38), and it clears both pIgA-R and DPP IV with the same kinetics (37). Therefore, we may reasonably speculate that such a mechanism is involved in the rapid clearance of DPP IV in SLE and SS patients, where low circulating levels of DPP IV are correlated with high titers of anti-DPP IV autoantibodies of the IgA class.

Soluble DPPIV/CD26 levels may be related to specific immunoregulatory patterns in autoimmune diseases. Recently, it was concluded that DPPIV/CD26 up-regulation may be suggestive of a new cellular pathway of Th1-like immune reactions (39,40). This effect would be in part attributable to a degradation of cytokines involved in Th2-like responses. Our study demonstrated decreased levels of soluble DPPIV/CD26 in SLE and in SS patients. These findings are concordant with presumed Th2-like immune responses diseases as it has been reported in SLE (41). In RA patient serum DPPIV/CD26 levels were within the normal range. There is accumulating evidence that T cells within RA synovium show mainly a Th1 pattern of cytokine production (42,43). Even though in RA patient serum DPPIV/CD26 levels were in the normal range, serum DPPIV activity was significantly decreased. These findings were correlated with DPP IV hypersialylation, and the enzyme activity was augmented after desialylation with V. cholerae neuraminidase.

It has been suggested that DPP IV lining the inner walls of blood vessels of most tissues may exert an anticoagulant activity by modifying the structure of fibrin monomers and preventing their degradation (44). Fibrin monomers can reduce the susceptibility of thrombin to inactivation by antithrombin-III (45).
As thrombin is chemotactic for both PMN cells and macrophages (46,47) this could form the basis for a pro-inflammatory feedback loop, which leads to recruitment of inflammatory cells into the joint, further in situ production of thrombin by these cells, and prolonged thrombin action due to the damage of serpins by free radical and proteolytic mechanisms (49, 50). In addition to a decrease in DPP IV activity, carboxypeptidase B is also decreased in the serum of RA patients (51). Both DPP IV and carboxypeptidase B regulate the formation and stability of the fibrin clot generated via the intrinsic pathway of coagulation (44,52). A decrease in the activity of both of these enzymes in RA would result in the enhancement in the rate of fibrin clot generation, augmenting plasmin generation at the site of inflammation. The increase in plasmin activity enhances the inflammatory response via cleavage of the C3 complement fraction, releasing C3a and fibrin split products, and also by monocyte recruitment into the inflamed tissue (53, 54).

In summary, our study suggests that the decrease in DPP IV activity in the sera of RA patients is associated with the hypersialylation of DPP IV, whereas in SLE and SS patients the decrease in such activity is caused by lower circulatory levels of the enzyme. Hypersialylation of DPP IV does not affect its activity in SLE patients; however, its reactivity with ADA is increased. The precise mechanism of hypersialylation remains to be elucidated.

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References
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