Comparison of Two Commercial ELISAs against an In-House ELISA for Measuring Soluble CD26 in Human Serum

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Background: CD26 is a T-cell costimulatory molecule with dipeptidyl peptidase IV (DPPIV) activity in its extracellular region. The relevance of sCD26 levels and disease activity has been reported in rheumatic or infectious disease. For certain metabolic and endocrine conditions, DPPIV inhibitors were recently developed as a new class of antidiabetic drugs that act by inhibiting DPPIV, the enzyme that inactivates incretin hormone. Higher levels of sCD26 in diabetic patients have been shown to be associated with a poor clinical response to DPPIV inhibitors, with sCD26/DPPIV being an adipokine that may impair insulin sensitivity. With the increasing use of serum sCD26 and DPPIV enzyme activity as biomarkers with potential clinical implications, accurate measurements of serum sCD26 levels and DPPIV enzyme activity are needed. **Methods:** We compare two commercially widely available and an in-house enzyme-linked immunosor-

bent assays (ELISAs) for measurement of serum sCD26 in healthy or diabetic human sera. **Results:** The significant discrepancies among the results obtained from commercially available and the in-house sCD26 assays were found. We also observed that a linear correlation between serum sCD26 level and DPPIV enzyme activity exists with the in-house ELISA, while the commercial ELISAs demonstrate a lack of consistency between serum sCD26 level and DPPIV enzyme activity. **Conclusion:** These data strongly suggest that new commercial assays for sCD26 plasma levels need detailed evaluation and validation with samples from clinically well-characterized patients, and results obtained from these newer assays should be compared to those obtained from well-established in-house assays such as our assay or other validated sCD26 ELISA assays. *J. Clin. Lab. Anal.* 29:106–111, 2015. © 2014 Wiley Periodicals, Inc.

Key words: CD26; dipeptidyl peptidase IV; T cell costimulation

INTRODUCTION

CD26 is a 110 kDa surface glycoprotein with dipeptidyl peptidase IV (DPPIV, EC 3.4.14.5) activity, being a serine protease that cleaves dipeptides from the N-terminus of peptides at the penultimate position (1). CD26 is also associated with T-cell signal transduction processes as a costimulatory molecule, as well as being a marker of T-cell activation (2). We have previously reported that effector CD26-mediated costimulatory activity is exerted via its DPPIV enzyme activity (2, 3). More recently, we have shown that serum-soluble CD26 (sCD26) and DPPIV

enzyme activity are inversely correlated with disease activity of patients with systemic lupus erythematosus (4).

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Other investigators have reported that serum sCD26 level was lower in rheumatoid arthritis (RA) patients than in healthy control or in patients with osteoarthritis (OA) (5, 6). sCD26 lacks transmembrane and cytoplasmic domains (the sequence starting at the 39th amino acid position of the full-length of CD26) (7), although its molecular weight is similar to that of the full-length transmembrane CD26 since it exists as a homodimer in serum/plasma and is heavily glycosylated between the 48 and 324th amino acid positions (8). Meanwhile, DPPIV enzyme activity has been widely studied in metabolic and endocrine disorders, and DPPIV inhibitors have been developed as a new class of antidiabetic drugs that act by inhibiting DPPIV, the enzyme that inactivates incretin hormone (9). For instance, higher serum level of sCD26 was reported to be associated with a worse response to sitagliptin in patients with type 2 diabetes (T2DM) controlled inadequately by metformin and/or sulfonylurea therapy (10). Moreover, other investigators have reported that sCD26/DPPIV is one of the adipokines that may impair insulin sensitivity (11). The potentially important role played by CD26/DPPIV in the clinical setting has led to rising interest in the quantification of serum sCD26 level and DPPIV enzyme activity over the past decade. This is also reflected in the number of publications on this topic and in the recent worldwide availability of commercial CD26 enzyme-linked immunosorbent assays (ELISAs), which can help researchers working in this area avoid the time-consuming process of in-house assay development and maintenance. However, it is our belief that these new commercial assays should be proven to deliver accurate, reliable, and reproducible data prior to usage for analysis of valuable patient-derived samples, even if the assay is certified by the manufacturer. In addition, DPPIV enzymatic activity has been correlated with sCD26 concentration (12), with the enzymatic activity of DPPIV being determined with the use of either chromogenic or fluorogenic substrates. Meanwhile, commercially available DPPIV activity assay kits have been shown to provide comparable results (13). On the other hand, comparative studies of different assay kits for sCD26 quantification have not yet been rigorously performed using human samples. The aim of this study is therefore to compare two commercially worldwide available sCD26 ELISA assays and our own in-house ELISA for detecting both sCD26 level and specific DPPIV enzyme activity.

MATERIALS AND METHODS

Serum Collections and Storage

Peripheral blood samples were collected from healthy adult volunteers and T2DM patients (regularly seen and treated at the Kobari General Hospital), using BD Vacu-

tainer blood collection tube SSTII (BD, Franklin Lakes, NJ). Serum was obtained from 5 ml whole blood by centrifugation at $1,500 \times g$ at 4°C for 10 min, and stored at -80°C in 500 μl aliquots. Human study protocols were approved by the Ethics Committees at the Kobari General Hospital (Authorization Number 10) and Juntendo University (Authorization Number 2012192). Informed consent was obtained from all patients. Patients' gender and age were matched between normal and T2DM groups. All studies on human subjects were conducted according to the principles set out in the Declaration of Helsinki.

ELISA Procedure

Serum samples were removed from the -80°C freezer and quickly thawed in a water bath at 37°C . All samples were centrifuged $3,000 \times g$ for 5 min to discard debris, and were then assayed in duplicate. Two commercial ELISA kits (Human sCD26 Platinum ELISA Kit, or Human DPPIV/CD26 Quantikine ELISA Kit) were purchased from eBioscience (Vienna, Austria), or R&D Systems, Inc. (Minneapolis, MN), respectively. Measurement of sCD26 level was performed according to the manufacturers' instructions. An in-house sandwich ELISA for sCD26 was established in our laboratory (14). Briefly, Maxisorp ELISA immunoplates (Nunc, Roskilde, Denmark) were coated with 100 μl of 10 $\mu\text{g}/\text{ml}$ anti-CD26 monoclonal antibody (mAb) (clone: 5F8, mouse IgG₁) in 0.05 M carbonate/bicarbonate buffer, pH 9.6, by incubating overnight at 4°C . Remaining binding sites were blocked with 200 μl of 25% StartingBlock Blocking Buffer (Thermo Scientific, South Logan, UT) for 2 h at room temperature. Plates were then incubated with 100 μl of serum appropriately diluted with 0.01 M phosphate buffered saline (PBS) containing 0.05% Tween 20 at 4°C overnight. Bound sCD26 molecules were detected by incubating with 100 μl of biotin conjugated anti-CD26 mAb (clone: 9C11, mouse IgG₁) followed by ExtrAvidin-alkaline phosphatase (Sigma-Aldrich, St. Louis, MO). The plates were developed with 1 mg/ml *p*-nitrophenyl phosphate in 10 mM diethanolamine buffer, pH 9.6, containing 0.5 mM MgCl_2 . Color development was monitored at 405 nm on an iMark Microplate Reader (Bio-Rad Laboratories, Hercules, CA). Absorbance data were analyzed using Microplate Manager 6 Software (Bio-Rad Laboratories). In parallel with sCD26 protein concentration, DPPIV enzyme activity of bound sCD26 to 5F8 was detected by incubation with glycylproline *p*-nitroanilide (Gly-Pro-*p*-NA, 1 mg/ml in PBS; Sigma-Aldrich). Color development was measured at 405 nm. These results were standardized by a standard curve for *p*-nitroaniline (Sigma-Aldrich). Recombinant CD26/DPPIV (R&D Systems, Inc.) for a standard curve was used as the identical reagent in all three assays. 5F8

TABLE 1. Details of ELISA Assays as Specified by ELISA Manufactures and In-House Protocols

	R&D Quantikine	eBioscience	In-house
Kit format	12 by 8-well strips (flat-bottom)	12 by 8-well strips (flat-bottom)	96-well plate (flat-bottom)
Capture antibody	Rat mAb (no further information)	mAb (no further information)	Mouse mAb (5F8), house-made
Sample dilution and preparation	100-fold dilution	Five-fold dilution	20-fold dilution
Volume/ELISA-well	150 µl	100 µl	100 µl
Dilution buffer	Buffered protein base with blue dye and preservative	No information	0.05% Tween 20-PBS
Sample incubation temperature	Room temperature	Room temperature	4°C
Sample incubation period	2 h	3 h	Overnight
Wash buffer	Buffered surfactant with preservative	1% Tween 20-PBS	0.05% Tween 20-PBS
Detection antibody	HRP conjugated pAb	Biotinylated mAb	Biotinylated mouse mAb (9C11)
Conjugate/substrate	Direct conjugation to detection pAb/TMB	Streptavidin-HRP/TMB	ExtrAvidin-ALP/PNPP
Optical density values (nm)	450	450	405
Standard ^a	Recombinant human DPPIV	Recombinant human DPPIV	Recombinant human DPPIV
Dynamic range (of Standard curve)	0.00031–0.020 (µg/ml)	0.0156–0.5 (µg/ml)	0.005–3.0 (µg/ml)
Data reduction	Log concentration of serial diluted standard against OD values	Log concentration of serial diluted standard against OD values	Log concentration of serial diluted standard against OD values
Precision (intra/interassay)	4–7.3%/8.2–9.1% (precision supplied by manufacturer)	0.8–13.7%/3.3–14.7% (precision supplied by manufacturer)	0.8–14.3%/4.1–15.9%

ALP, alkaline phosphatase; HRP, horseradish peroxidase; PBS, phosphate buffered saline; PNPP, *p*-nitrophenyl phosphate; TMB, tetramethylbenzidine.

^aRecombinant human DPPIV is composed of the 34–766th amino acids of human CD26/DPPIV, with a C-terminal 6xHis tag, produced with the use of mouse myeloma cells NS0. This human DPPIV is highly glycosylated and shows 115 kDa in reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and presents as a homodimer in nonreducing condition.

was produced by immunizing BALB/cJ mice with a phytohemagglutinin-stimulated T cell line derived from the New World primate species *Aotus trivirgatus* (15). 9C11 was produced by immunizing mice with murine pre-B 300–19 cell line of human CD26 transfectant (16). Selected details for the three sCD26 assays including assay precisions are summarized in Table 1.

Intra-assay and Interassay Variations

To analyze for intra-assay variation, three replicates of three different samples were run in one assay. To analyze for interassay variation, three different samples were run in three independent assays. The intra-assay or interassay variations were calculated using the following formula:

$$(\text{SD of assay results})/(\text{mean of assay results}) \times 100(\%).$$

Statistical Analysis

The values measured are shown as mean $\pm$ standard deviation (SD). The differences between groups were analyzed by one-way repeated measures ANOVA. 95% CIs were calculated and *P* values ≤ 0.05 were considered sta-

tistically significant. Correlation was calculated as Pearson product-moment correlation coefficient. Statistical analysis was performed using Microsoft Excel 2011 (Microsoft Corp., Redmond, WA) or GraphPad PRISM version 6 (GraphPad Software Inc., La Jolla, CA).

RESULTS AND DISCUSSION

As shown in Figure 1A, serum sCD26 concentrations of healthy adults ($n = 12$) measured by the two commercial kits and the in-house assay differed in all samples by approximately fourfold (0.34 ± 0.14 µg/ml or 0.36 ± 0.15 µg/ml vs. 1.20 ± 0.31 µg/ml). The values measured by our in-house assay were significantly higher than those by the R&D Quantikine assay ($P < 0.0001$ [95% CI, 0.65–1.03]), or eBioscience Platinum assay ($P < 0.0001$ [95% CI, 0.63–1.02]) (Fig. 1A). It has been reported that DPPIV enzymatic activity has been correlated with the concentration of sCD26 using the normal human sera (12). We therefore evaluated for potential correlation between DPPIV enzymatic activity and sCD26 level in the serum samples described above. For this purpose, we performed our in-house capture assay method using anti-human CD26 mAb as a capture antibody for detecting DPPIV specific

Fig. 1. Comparison of 12 normal serum samples, tested with three different ELISA assays for sCD26. (A) sCD26 levels in serum samples of 12 healthy adults were measured using the R&D Quantikine (solid squares), eBioscience Platinum (solid triangles), or in-house ELISA assays (solid circles). Each dot indicates individual value. Data of same samples are connected with horizontal lines. (B) Correlation between levels of sCD26 and DPPiV enzyme activity in serum samples of 12 healthy adults. (a) Comparison between in-house and the R&D Quantikine ELISA assays. (b) Comparison between in-house and the eBioscience Platinum ELISA assays. DPPiV enzyme activity was measured using our in-house capture method as described in Materials and Methods. (C) DPPiV enzyme activity in serum samples of 12 healthy adults was measured using the R&D Quantikine (solid squares), eBioscience Platinum (solid triangles), or in-house ELISA plates (solid circles). The samples were prepared and applied to each plate by the same method as the in-house method. DPPiV enzyme activity was measured using our in-house capture method as described in Materials and Methods. Data of same samples are connected with horizontal lines. N.S. denotes not significant.

to sCD26. It is conceivable that 5F8 is used for capturing sCD26 without affecting DPPiV enzyme activity in the plate since the epitope of human CD26 recognized by 5F8 is in the extracellular region of the truncated human CD26 containing the N-terminal residues 1–449th lacking DPPiV enzyme activity (16). Moreover, 5–10% of DPPiV activity in normal plasma/serum has been reported to be associated with other DPPiV-like peptidases such as DASH (DPPiV activity and/or structure homologues) proteins (17). Therefore, it is also possible to correlate the DPPiV activity with sCD26 level by capturing sCD26 molecules from the samples. As shown in Figure 1B, levels of sCD26 measured by our in-house assay clearly correlated with DPPiV enzyme activity ($r^2 = 0.86$; slope, 0.08 ± 0.002) (solid circles in panels a or b), while levels of sCD26 measured by the R&D Quantikine (solid squares in panel a) or eBioscience Platinum assays (solid triangles in panel b) did not correlate with DPPiV enzyme activity ($r^2 = 0.12$; slope, 0.02 ± 0.003 , or $r^2 = 0.01$;

slope, 0.02 ± 0.003 , respectively). To exclude the possibility that the incubation period of samples may influence the results, assays for sCD26 using the R&D Quantikine or eBioscience Platinum kits were conducted in which the sample incubation period in the capture step was extended to overnight at 4°C , which was the same method as in our in-house sCD26 assay. However, the obtained values were not influenced by extending the incubation period in the capture step (data not shown). Moreover, it was important to determine whether the values for DPPiV enzyme activity measured by the in-house plate were equal to those obtained from the commercial plates. To address this point, we evaluated captured DPPiV enzyme activity on the plates coated with the capture antibody in the R&D Quantikine or eBioscience Platinum kits. As shown in Figure 1C, the values of the captured DPPiV enzyme activity were similar for each assay. These results suggest that the ability to capture sCD26 is similar among the two commercial and our in-house plates, and

Fig. 2. Comparison of 50 serum samples of patients with T2DM, tested with three different ELISA assays for sCD26. (A) sCD26 levels in serum samples of 50 T2DM adults were measured using the R&D Quantikine (solid squares), eBioscience Platinum (solid triangles), or in-house ELISA assays (solid circles). Each dot indicates individual value. Data of same samples are connected with horizontal lines. N.S. denotes not significant. (B) Correlation between levels of sCD26 and DPPIV enzyme activity in serum samples of 50 T2DM patients. (a) Comparison between in-house and the R&D Quantikine ELISA assays. (b) Comparison between in-house and the eBioscience Platinum ELISA assays. DPPIV enzyme activity was measured using our in-house capture method as described in Materials and Methods.

that the observed variability among the assays may be due to differences in the sCD26 detection process.

We next determined serum levels of sCD26 in T2DM patients who were not being treated with DPPIV inhibitor therapy ($n = 50$). As shown in Figure 2A, serum sCD26 concentrations of T2DM patients measured by the two commercial and our in-house assays differed in all samples by approximately four- to fivefold ($0.30 \pm 0.12 \mu\text{g/ml}$ or $0.40 \pm 0.10 \mu\text{g/ml}$ vs. $1.45 \pm 0.34 \mu\text{g/ml}$). The two commercial assays measured samples of T2DM patients in the range of $0.15\text{--}0.74 \mu\text{g/ml}$ or $0.19\text{--}0.74 \mu\text{g/ml}$ (Fig. 2A), values that were compatible with the previously reported range of serum sCD26 concentrations of T2DM patients, using commercial assays (10, 18). As observed in healthy samples (Fig. 1A), the values measured by our in-house assay were significantly higher than those by the R&D Quantikine assay ($P < 0.0001$ [95% CI, 1.04–1.27]) or eBioscience Platinum assay ($P < 0.0001$ [95% CI, 0.91–1.19]) (Fig. 2A). Moreover, levels of sCD26 measured by our in-house assay clearly correlated with DPPIV enzyme activity ($r^2 = 0.94$; slope, 0.08 ± 0.001) (solid circles in panels a or b of Fig. 2B), while levels of sCD26 measured by the R&D Quantikine (solid squares in panel a of Fig. 2B) or eBioscience Platinum (solid triangles in panel b of Fig. 2B) assays did not correlate with DPPIV enzyme activity ($r^2 = 0.43$; slope, 0.02 ± 0.001 , or $r^2 = 0.002$; slope, 0.02 ± 0.001 , respectively). These data indicated that serum sCD26 concentration in human samples may be underestimated by the commercially available sCD26 assay, and that poor linearity between DPPIV enzyme activity and sCD26 level may misinterpret the clinical relevance of this molecule.

A critical issue for ELISAs is that the standard curve is stably plotted among different assays. In the present as-

says, we used commercially available recombinant human DPPIV derived from the same lot as the standard compound for all three ELISAs (Table 1), and obtained almost identical standard curves among various assays (data not shown). Therefore, the differences seen in sCD26 levels between the two commercial and our in-house ELISAs were likely not to be due to differences in the quality of the standard. To exclude the possibility that the clones of the capture antibody may influence the results, the in-house DPPIV enzyme activity assay was conducted with 1F7 as a capture antibody, a different epitope from 5F8. However, the obtained values were not influenced by the different epitope capture antibody (data not shown), suggesting higher validity of the in-house DPPIV enzyme activity assay. Moreover, it is theoretically possible that single nucleotide polymorphisms (SNPs) may affect the reactivity of the sandwich antibody sets of the commercial kits. One nonsynonymous coding region SNP Arg492Lys (G→A, exon 18) was reported, although its frequency or functional significance has not yet been determined (19). Since information regarding the clones of the capture and detection antibodies or the concentrations of these antibodies used in the commercial assays is not publicly available, the exact reasons for the observed inconsistencies seen with the commercial assays evaluated in this article are not clear. The epitopes of the anti-human CD26 mAbs used in our in-house assay are distinct from each other (16), that is, 5F8 or 9C11 recognizes 359–449th or 740–766th amino acids of human CD26, respectively. Moreover, these epitopes are also distinct from that of the humanized anti-CD26 mAb, which was developed in our laboratory for clinical uses, such as in the potential treatment of malignant methothelioma, or graft-versus-host disease (20, 21). Therefore, our in-house sCD26 assay can

be used to detect sCD26 or DPP-IV enzyme activity as biomarkers of patients who are subjected to humanized anti-CD26 mAb therapy.

CONCLUSIONS

Given emerging data indicating the importance of serum levels of sCD26 in certain clinical settings, there is an urgent need for precise measurement and correlation of values obtained from different assays. In light of the discrepancies noted with results obtained from commercially available sCD26 assays in the current study, it seems unlikely that the established associations between sCD26 plasma levels and diseases such as T2DM, RA, OA, chronic infection, or malignant diseases can be reliably reproduced by the commercial assays (1, 10, 17). On the other hand, in view of the linearity observed between sCD26 level and DPP-IV enzyme activity, our in-house assay appears to be superior to the commercial assays as a means to evaluate sCD26/DPP-IV as clinically relevant biomarkers. The current study strongly suggests that new commercial assays for sCD26 plasma levels need detailed evaluation and validation with samples from clinically well-characterized patients, and results obtained from these newer assays should be compared to those obtained from well-established in-house assays such as our assay or other validated sCD26 ELISA assays.

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CONFLICT OF INTEREST

The authors declare no competing financial interests associated with this article.

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