

Reduced expression of dipeptidyl peptidase (DPP) IV in peripheral blood T lymphocytes of oral cancer patients

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To investigate the mechanism of decrease of serum DPP IV activity in oral cancer patients, we analyzed the expression of DPP IV in peripheral blood T lymphocytes of oral cancer patients and healthy subjects. Consequently, serum DPP IV activity was found to correlate significantly with the number of peripheral blood lymphocytes (PBL), T lymphocytes and CD26 (identified as DPP IV)⁺ T lymphocytes in healthy subjects, and the number of PBL and CD26⁺ T lymphocytes in cancer patients. However, the numbers of PBL and T lymphocytes were significantly less in cancer patients than in healthy subjects. Although the number of CD26⁺ T lymphocytes was somewhat greater in cancer patients than in healthy subjects, serum DPP IV activity was significantly lower in cancer patients. DPP IV activity and amount of CD26 in T lymphocyte plasma membranes were much less in cancer patients than in healthy subjects. These findings suggest that a decrease in the number of T lymphocytes and the small amount of DPP IV in their plasma membrane may contribute to the decrease of serum DPP IV activity in cancer patients.

Key words: CD26; lymphocyte subpopulations; oral cancer; serum DPP IV activity

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Dipeptidyl peptidase (DPP) IV (EC3.4.14.5) is a membrane-bound enzyme that hydrolyzes N-terminal X-proline from peptides (1, 2). In previous studies we reported that DPP IV activity in the sera of oral cancer patients was significantly decreased as compared with that of healthy subjects, and it changed dynamically as a reflection of clinical status during cancer therapies (3). Therefore, serum DPP IV activity was suggested to be a possible marker of oral cancer. Since this enzyme activity was decreased even in patients with malignant tumors other than oral cancer, it was considered to be a nonspecific tumor-burden marker. However, the precise mechanism of the decrease of serum DPP IV activity in cancer patients is unclear. Recently, it has been shown that DPP IV is CD26, a surface

antigen of T lymphocytes, and that it plays a role in the process of proliferation of T lymphocytes (4). In this study, therefore, the expression of DPP IV in peripheral blood T lymphocytes of oral cancer patients was analyzed in comparison with that of healthy subjects.

Material and methods

Blood samples

Heparinized blood samples were collected from 22 newly diagnosed and untreated oral cancer patients (male:female=13:9; age 25-91 years; average 62.0 years) and 26 healthy subjects (male:female=15:11; age 26-79 years; average 50.3 years). The oral cancer patients all were histologically diagnosed as squamous cell carcinoma and were composed of 4 people in Stage I, 7 in

Stage II, 6 in Stage III and 5 in Stage IV in the TNM classification of the International Union Against Cancer (UICC) system (1987). The number of samples in each experiment was different because of limitations of the sample volume obtained.

Assay for lymphocyte subpopulations by flow cytometry

Lymphocyte subpopulations (CD3, CD4, CD8 and CD26) were analyzed by flow cytometry using monoclonal antibodies (anti-human Leu-4, Leu-3a, Leu-2a and Ta1). Phycoerythrin (PE)-labelled anti-human Leu-4, Leu-3a and Leu-2a mouse monoclonal antibodies were purchased from Becton-Dickinson, San Jose, CA, USA, and fluorescein isothiocyanate (FITC)-conjugated

anti-human Ta1 mouse monoclonal antibody was from Coulter Immunology, Hialeah, FL, USA. One hundred μ l of heparinized blood was mixed with 20 μ l of PE-labelled and/or FITC-conjugated antibody and left to stand at 4°C for 30 min. After that, 1.4 ml of hemolysis solution (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA-2Na) was added, gently shaken and left to stand at room temperature for 5 min. After centrifugation at 300 \times g for 5 min, sedimented lymphocytes were washed three times with Ca²⁺- and Mg²⁺-free phosphate buffered saline (PBS) and suspended in 0.2 ml PBS. The samples were measured by single or two color analysis for PE-stained and FITC-stained lymphocytes by a flow cytometer (FACScan, Becton-Dickinson, San Jose, CA, USA) with an argon ion laser (488 nm).

Separation of T lymphocytes and preparation of their plasma membranes

Peripheral blood lymphocytes (PBL) were isolated by centrifugation at 800 \times g for 20 min using Lymphoprep™ (Nycomed Pharma As, Oslo, Norway). After washing three times with PBS, lymphocytes were suspended at a density of 1 \times 10⁷ cells/ml in heat-inactivated fetal bovine serum (FBS, Filtron Pty Ltd., Dooklyn, Australia). Sheep red blood cells (SRBC) were treated with 4% 2-aminoethylisothiouronium (AET, Wako Pure Chemical Industries, Ltd., Osaka, Japan) solution (pH 9.0) at 37°C for 15 min and were suspended at a density of 2 \times 10⁹ cells/ml in heat-inactivated FBS. Lymphocyte suspension was mixed with the same volume of AET-treated SRBC suspension, centrifuged at 200 \times g for 5 min and left to stand for 2 h in an ice bath to allow rosette formation (E-rosette). T lymphocyte formed E-rosettes were collected by low speed centrifugation with Lymphoprep, and SRBC were dissolved in hemolysis solution. T lymphocyte samples obtained were ascertained to be composed of over 95% CD3⁺ by staining with PE-labelled anti-human Leu-4 antibody.

T lymphocyte plasma membranes were prepared by the method of MENTLEIN *et al.* (5). T lymphocytes were suspended at a density of 1 \times 10⁷ cells/ml in cell lysis buffer (75 mM KCl, 65 mM NaCl, 10 mM Hepes, pH 7.4) and disrupted in a cell disruption bomb (No. 4639, Parr Instrument, Moline, IL, USA) under inner pressure of 50 atmospheres with nitro-

gen gas. The disrupted cell suspension was centrifuged at 10,000 \times g for 15 min at 4°C and the supernatant was collected. The plasma membrane was pelleted as a microsomal fraction by ultracentrifugation at 105,000 \times g for 2 h and was solubilized in 50 mM Tris-HCl buffer (pH 7.4) containing 1% Triton X-100.

Protein measurement

Protein concentrations were measured by the modified method of LOWRY (6) with the use of bovine serum albumin (BSA) as a standard.

Assay for DPP IV activity

DPP IV activity was determined by the fluorometric method of KATO *et al.* (7) in which glycylproline methylcoumarinamide (Gly-Pro-MCA) tosylate (Peptide Institute, Protein Research Foundation, Osaka, Japan) was used as the substrate. The incubation mixture (100 μ l) contained the following: 40 μ l of 150 mM glycine-NaOH buffer (pH 8.7), 25 μ l of 2 mM substrate, 25 μ l of distilled water and 10 μ l of enzyme sample. After incubation at 37°C for 30 min, the reaction was terminated by the addition of 1 ml of 1 M acetate buffer (pH 4.0). The fluorescence intensity of 7-amino-4-methylcoumarin liberated was measured at 460 nm with excitation

at 380 nm by a spectrofluorophotometer (RF-540, Shimadzu Corporation, Kyoto, Japan). One international unit (IU) of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 μ mol of 7-amino-4-methylcoumarin/min/liter of serum at 37°C. The DPP IV activity of T lymphocyte plasma membrane was expressed by nmol/min/mg protein as specific activity.

Detection of CD26 antigen by Western immunoblotting

Twenty micrograms of solubilized T lymphocyte plasma membrane were submitted to SDS-polyacrylamide gel electrophoresis (PAGE) in 7.5% acrylamide resolving gels. The electrophoretic transfer of proteins from SDS-PAGE to polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore Corporation, Bedford, MA, USA) was performed according to the modification of the method of TOWBIN *et al.* (8). After transfer the membrane was wetted in methanol for 3 s and immersed in water and then transfer buffer (100 mM Tris pH 8.3, 192 mM glycine, 5% methanol) to remove methanol; transfer was done at 2 mA/cm² constant for 1 h in transfer buffer. The PVDF membrane was then followed by a blocking step in TBS (50 mM Tris-

Table 1. The number of PBL and lymphocyte subpopulations in oral cancer patients and healthy subjects

	Healthy subjects ^a	Cancer patients ^a	Ratio ^b	Difference ^c
PBL per μ l	2,456 \pm 746 (n=26)	1,257 \pm 638 (n=22)	0.51	P<0.0001
T(CD3 ⁺) lymphocytes per μ l	1,660 \pm 562 (n=26)	821 \pm 368 (n=16)	0.49	P<0.0001
Ratio of CD3 ⁺ cells to PBL	67.6%	65.3%		
CD4 ⁺ cells per μ l	892 \pm 404 (n=13)	525 \pm 282 (n=12)	0.59	P<0.02
Ratio of CD4 ⁺ cells to PBL	36.3%	41.8%		
CD8 ⁺ cells per μ l	646 \pm 262 (n=13)	438 \pm 135 (n=12)	0.68	N.S.
Ratio of CD8 ⁺ cells to PBL	26.3%	34.8%		
CD3 ⁻ cells per μ l	796 \pm 258 (n=26)	467 \pm 200 (n=16)	0.59	P<0.001
Ratio of CD3 ⁻ cells to PBL	32.4%	36.3%		
CD3 ⁺ CD26 ⁺ cells per μ l	34 \pm 41 (n=26)	70 \pm 70 (n=16)	2.06	N.S.
CD3 ⁻ CD26 ⁺ cells per μ l	8 \pm 11 (n=26)	11 \pm 11 (n=16)	1.38	N.S.

Lymphocyte subpopulations were analyzed by flow cytometry using PE-labelled anti-human Leu-4, Leu-3a, Leu-2a mouse monoclonal antibodies (Becton-Dickinson, San Jose, CA, USA) and/or FITC-conjugated anti-human Ta1 mouse monoclonal antibody (Coulter Immunology, Hialeah, FL, USA).

^a mean \pm SD.

^b Ratio of the value in oral cancer patients to that in healthy subjects.

^c P values were determined by using the Mann-Whitney U-test.

N.S.: not significant

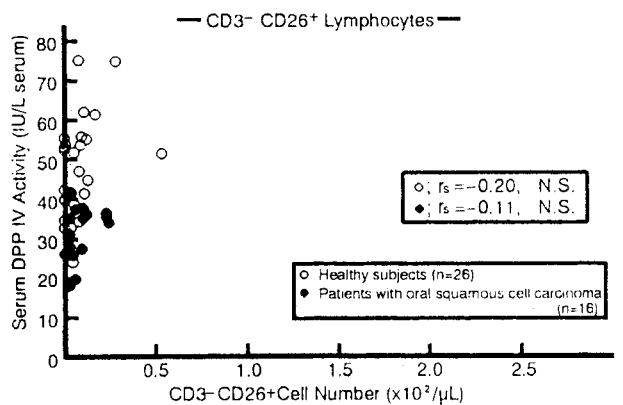
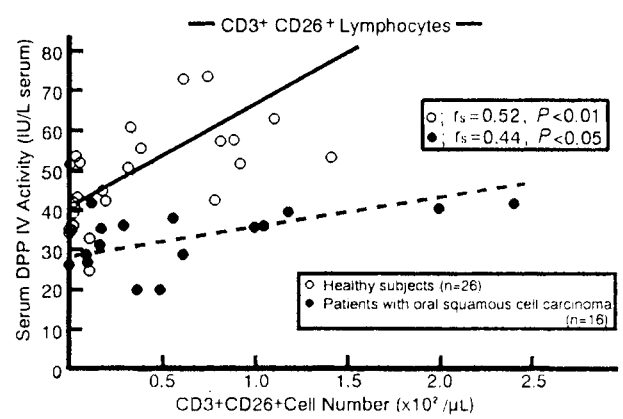
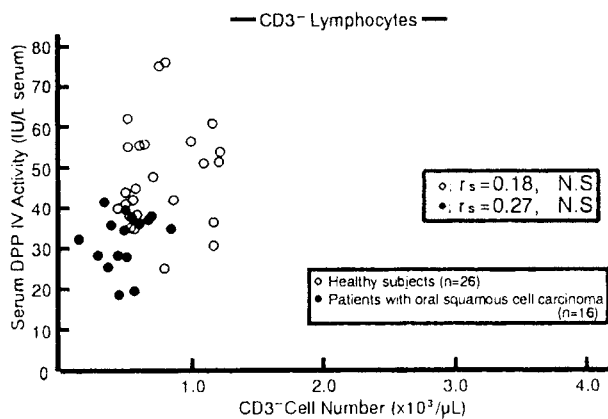
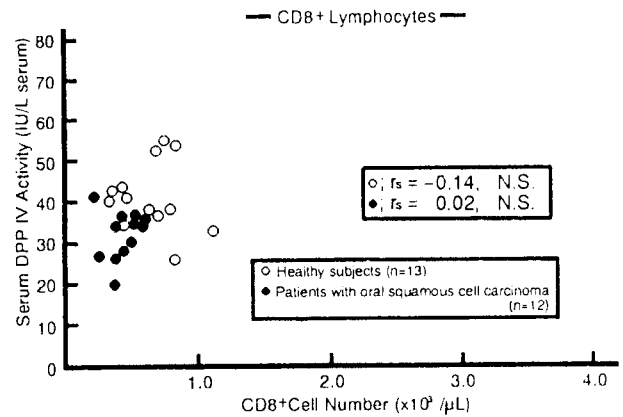
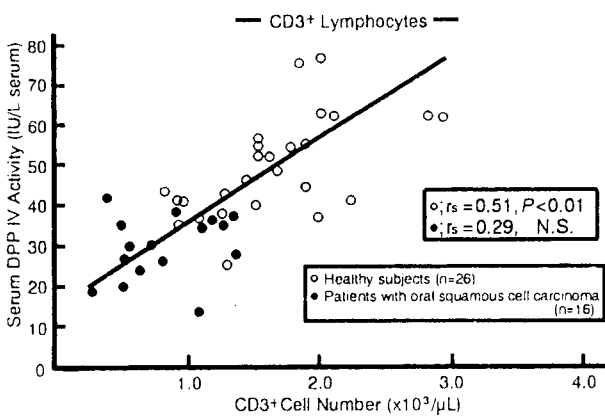
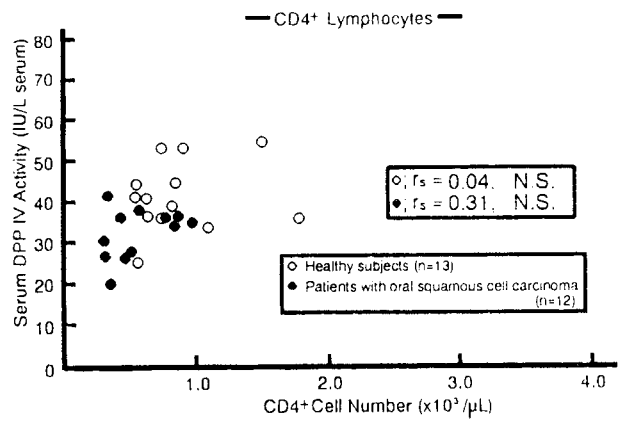
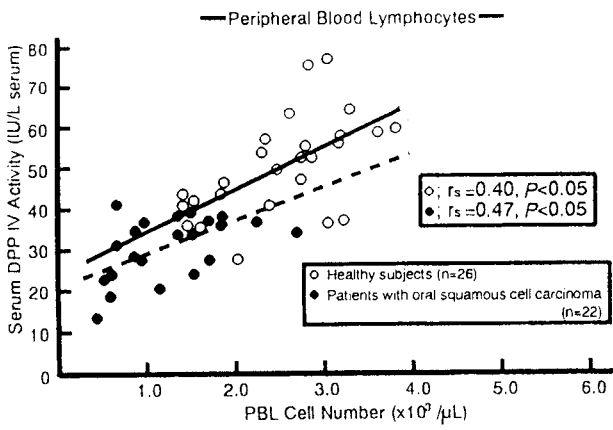


Fig. 1. Relationship between the number of PBL and lymphocyte subpopulations and serum DPP IV activity in oral cancer patients and healthy subjects. The solid line indicates the regression line in healthy subjects and the dotted line is that in cancer patients. r_s : Spearman's rank correlation coefficient.

HCl pH7.6, 150 mM NaCl) containing 5% BSA for 1 h at room temperature. The membrane was incubated with a 500-fold dilution of anti-human Ta1 mouse monoclonal antibody for 2 h. After washing with TBS containing 0.05% Tween-20 (TBST), the membrane was further incubated with a 500-fold dilution of biotinylated anti-mouse IgG rabbit antiserum (Tago Immunologicals, Burlingame, CA, USA) for 1 h at room temperature and then rinsed with TBST. For the color reaction, the membrane was incubated with alkaline phosphatase-labelled StreptABCComplex (Dakopatts a/s, Denmark) for 20 min at room temperature and visualised with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma Chemical Co., St. Louis, MO, USA). The relative density of the protein bands was measured by a densitometer (AE-6900, Atto Corporation, Tokyo, Japan).

Statistical analysis

Statistical analysis was performed by using the Mann-Whitney U-test and Spearman's rank correlation coefficient (r_s).

Results

Comparison of lymphocyte subpopulations in peripheral blood of cancer patients with those of healthy subjects

The number of peripheral blood lymphocytes (PBL) and lymphocyte subpopulations obtained by flow cytometry was compared between cancer patients and healthy subjects. The number of PBL, T lymphocytes ($CD3^+$ cells), $CD4^+$ (helper/inducer) and non-T lymphocytes ($CD3^-$ cells) was about two-fold less in cancer patients than in healthy subjects ($P < 0.02-0.0001$). However, the ratio of T lymphocytes, non-T lymphocytes and $CD4^+$ to PBL did not show much difference between them. The $CD26$ (DPP IV) $^+$ cells in peripheral blood T lymphocytes ($CD3^+$) and non-T lymphocytes ($CD3^-$) were unexpectedly few, but the number of $CD26^+$ T lymphocytes was two-fold more in cancer patients than in healthy subjects (Table 1).

Relationship between lymphocyte subpopulations and serum DPP IV activity

The activity of serum DPP IV was significantly decreased in the 22 cancer pa-

tients (29.9 ± 7.4 IU/l serum) compared with the 26 healthy subjects (47.8 ± 12.4 IU/l serum) ($P < 0.001$). Therefore, we examined whether or not the number of PBL and lymphocyte subpopulations correlate with serum DPP IV activity (Fig. 1). Consequently, serum DPP IV activity was found to correlate significantly with the number of PBL ($P < 0.05$), T ($CD3^+$) lymphocytes ($P < 0.01$) and $CD26^+$ T lymphocytes ($P < 0.01$) in healthy subjects, and with the number of PBL ($P < 0.05$) and $CD26^+$ T lymphocytes ($P < 0.05$) in cancer patients. The number of non-T ($CD3^-$) lymphocytes, $CD4^+$ and $CD8^+$ lymphocytes and $CD26^+$ non-T lymphocytes showed no correlation with serum DPP IV activity (Fig. 1).

Relationship between DPP IV activity in plasma membrane of peripheral blood T lymphocytes and serum DPP IV activity

Since the number of peripheral blood T lymphocytes and $CD26^+$ T lymphocytes was found to correlate significantly with serum DPP IV activity, we next examined DPP IV activity in the plasma membranes of peripheral blood T lymphocytes because $CD26^+$ T lymphocytes were very few and it was difficult to assay the enzyme activity. When DPP IV activity in the microsomal fraction isolated from peripheral blood T lymphocytes was measured in 15 cancer patients (age 25–91 years, average 61.4 years) and 22 healthy subjects (age 26–69 years, average 44.0 years), DPP IV activity in T lymphocyte plasma membranes demonstrated a significant correlation with serum DPP IV activity ($P < 0.001$) in healthy subjects but not in cancer patients (Fig. 2), and it was 3.4-fold less in cancer patients than in healthy subjects (Table 2).

Decreased amount of CD26 (DPP IV) in plasma membrane of peripheral blood T lymphocytes in cancer patients

To determine the amount of DPP IV enzyme protein in T lymphocyte plasma membrane, Western immunoblotting using anti-CD26 antibody, Ta1, was carried out. The CD26 protein bands were detected clearly at the molecular weight of 110 kDa. The protein bands in three cancer patients were thinner than those in three healthy subjects, and were about half the amount as measured by a densitometer (Fig. 3).

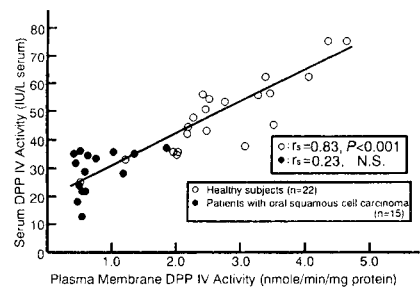


Fig. 2. Relationship between DPP IV activity in T lymphocyte plasma membranes and serum DPP IV activity in oral cancer patients and healthy subjects. The solid line indicates the regression line in healthy subjects. r_s : Spearman's rank correlation coefficient.

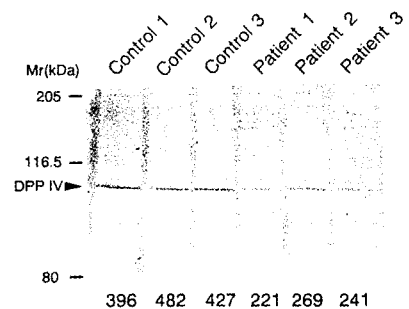


Fig. 3. Detection of CD26 antigen (DPP IV) in plasma membrane of peripheral blood T lymphocytes in oral cancer patients and healthy subjects by Western immunoblotting. Twenty μ g of T lymphocyte plasma membrane were submitted to SDS-PAGE, and CD26 antigen (DPP IV) was detected by Western immunoblotting with anti-human Ta1 mouse monoclonal antibody. Figures below the lanes indicate the relative density measured by a densitometer.

Discussion

It has been reported that serum DPP IV activity was significantly decreased in patients with malignant tumors, except for hepatoma. In oral cancer patients, we ascertained the same facts (3). Furthermore, we reported that a decrease in serum DPP IV activity occurred from the early stage of hamster buccal pouch carcinogenesis with 9, 10-dimethyl-1,2-benzanthracene (9). These studies suggest that serum DPP IV activity could become an important marker in the diagnosis of carcinoma and estimation of the prognosis of patients, even though it is a non-specific tumor-burden marker. However, the mechanism that brings about the decrease in DPP IV activity in sera of cancer patients is still unclear. At least, three facts have been found: 1) DPP IV inhibitor was not detected in homogenates of carcinoma tissues,

Table 2. DPP IV activities in plasma membranes of peripheral blood T lymphocytes and in sera of oral cancer patients and healthy subjects

	DPP IV Activities (mean±SD)		Difference ^a
	Healthy subjects (n=22)	Cancer patients (n=15)	
T lymphocyte plasma membranes (nmol/min/mg protein)	2.7±1.0	0.8±0.4	P<0.0001
Serum (IU/l)	48.2±12.7	29.2±7.4	P<0.0001

T lymphocytes were isolated from PBL by low speed centrifugation in Lymphoprep™ (Nycomed Pharma As, Oslo, Norway) using rosette formation with AET-treated SRBC. T lymphocyte plasma membranes were prepared by the method of MENTLEIN *et al.* (5). T lymphocytes were disrupted by a cell disruption bomb (Parr Instrument, Moline, IL, USA), centrifuged at 10,000×g for 15 min at 4°C and then ultracentrifuged at 105,000×g for 2h at 4°C. The plasma membranes were collected as a microsomal fraction.

^a P values were determined by using the Mann-Whitney U-test.

cultured carcinoma cells, and sera of cancer patients (10); 2) the enzyme activity was decreased in carcinoma tissues (11) and cultured carcinoma cells (10) as compared to the normal counterparts; 3) DPP IV enzyme protein was reduced in sera of oral cancer patients as compared to the normal subjects (12), but these facts did not give complete understanding of the reduction of serum enzyme levels.

LOJDA *et al.* (13) found the presence of DPP IV activity in human T lymphocytes by using glycyproline-4-methoxy-2-naphthylamide as the substrate. SCHÖN *et al.* (14) reported that inhibitors and antibodies against DPP IV suppressed lymphocyte proliferation induced by mitogenic lectins (phytohemagglutinin, concanavalin A, pokeweed mitogen) and markedly reduced immunoglobulin production by pokeweed mitogen-stimulated lymphocytes as a consequence of impaired T cell function; this suggests that DPP IV is involved in human T lymphocyte activation. At the 4th International Workshop on Human Leucocyte Differentiation Antigens, the surface antigen of activated T lymphocytes that is recognized by the monoclonal antibodies, 134-2C2 and TS 145, was found to be DPP IV and named as CD26 (4). Since it is well-known that cancer patients are in the immunosuppressive state, as indicated by the decrease in the number of PBL and in lymphocyte function, especially in T lymphocytes, it is speculated that suppressive changes in T lymphocyte number and function cause the reduced expression of CD26 antigen (DPP IV) and result in the decrease of serum DPP IV activity. Therefore, we studied the expression of CD26 (DPP IV) in peripheral blood T lymphocytes of oral cancer patients in comparison

with that of healthy subjects to investigate the mechanism of decrease of serum DPP IV activity in cancer patients.

The flow cytometric analysis for PBL and lymphocyte subpopulations revealed that PBL, T lymphocytes, CD4⁺ T lymphocytes and non-T lymphocytes were significantly decreased in cancer patients as compared to healthy subjects (Table 1); also, the number of PBL and T lymphocytes in healthy subjects and of PBL in cancer patients, correlated with serum DPP IV activity (Fig. 1). Although it was found that CD4⁺ and CD8⁺ cells did not correlate with serum DPP IV activity in this study, SCHOLZ *et al.* (15) reported that 71% of DPP IV-positive T lymphocytes were CD4⁺ cells and 22% were CD8⁺ cells. The number of CD26 (DPP IV)⁺ T lymphocytes was unexpectedly low, and was 34 cells/μl in healthy subjects and 70 cells/μl in cancer patients on average. When the relationship between CD26⁺ T lymphocyte number and serum DPP IV activity was examined, a significant correlation was demonstrated in both healthy subjects (P<0.01) and in cancer patients (P<0.05) (Fig. 1). Measurement of DPP IV activity and amount of DPP IV (CD26) enzyme protein in plasma membrane of peripheral blood T lymphocytes demonstrated 3.4-fold less activity (Table 2) and 2-fold less amount (Fig. 3) in cancer patients than in healthy subjects. These results indicated that not only DPP IV activity but also the amount of DPP IV (CD26) enzyme protein was decreased in T lymphocyte plasma membranes of cancer patients. Therefore, it may be suggested that although the number of CD26⁺ T lymphocytes was two-fold more in cancer patients than in healthy subjects, DPP activity in those lymphocytes of cancer patients was lower than that of healthy subjects. Since DPP IV

is suggested to be involved in T cell activation, the higher number of CD26⁺ T lymphocytes in cancer patients than in healthy subjects may indicate compensation for impaired T cell function in cancer patients.

The origin of DPP IV enzyme in serum remains unknown. Since it is known that antigens existing on the surfaces of normal and cancer cells are released from the cell surface to serum or medium by shedding (16), it is considered that serum DPP IV activity is derived from a variety of cells or tissues. HINO *et al.* (17) reported that concomitant with the elevation of serum DPP IV activity in rats with hepatitis induced by carbon tetrachloride, DPP IV activity in the liver was decreased, suggesting that increased DPP IV in serum was derived from the liver. Although the liver is one of the largest organs in the body and has an important role in the origin of serum DPP IV, the direct involvement of the liver in the decrease of serum DPP IV activity in patients with malignant tumors, including oral cancer, seems unlikely. Although further investigation is necessary to elucidate the precise mechanism, we have suggested in the present study that reduced expression of DPP IV in T lymphocyte plasma membranes is an important factor in the decrease of serum DPP IV activity in oral cancer patients.

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