

Decreased expression and release of dipeptidyl peptidase IV (CD26) in cultured peripheral blood T lymphocytes of oral cancer patients

Takashi Uematsu¹, Masahiro Urade² and Minoru Yamaoka¹

¹Oral and Maxillofacial Surgery Department II, Matsumoto Dental College, Nagano, and
²Department of Dentistry and Oral Surgery, Hyogo College of Medicine, Hyogo, Japan

Uematsu T, Urade M, Yamaoka M: Decreased expression and release of dipeptidyl peptidase IV (CD26) in cultured peripheral blood T lymphocytes of oral cancer patients. J Oral Pathol Med 1998; 27: 106-10. © Munksgaard, 1998.

To investigate the mechanism whereby serum dipeptidyl peptidase (DPP) IV activity in oral cancer patients is decreased, we examined the expression of cell surface DPP IV, also known as CD26, in cultured peripheral blood T lymphocytes of these patients and the amounts of DPP IV released into culture medium; values were compared with those found in healthy subjects. When peripheral blood T lymphocytes were cultured in the presence of phytohemagglutinin, concanavalin A and/or interleukin-2, the proliferative response and expression of CD26 (DPP IV) in their plasma membranes were greatly diminished in oral cancer patients as compared with those in healthy subjects. In addition, DPP IV activity in lymphocyte culture medium was reduced more in oral cancer patients than in healthy subjects, indicating decreased shedding of DPP IV from activated T lymphocytes in the patients. Based on these findings, it is suggested that suppression of DPP IV expression in peripheral blood T lymphocytes is one of the important factors involved in the mechanism of decrease of serum DPP IV activity in oral cancer patients.

Key words: CD26; DPP IV activity; lymphocyte culture; oral cancer

Masahiro Urade, Department of Dentistry and Oral Surgery, Hyogo College of Medicine, 1-1, Mukogawa-cho, Nishinomiya, Hyogo 663, Japan

Accepted for publication December 12, 1997

Dipeptidyl peptidase IV (DPP IV, EC 3.4.14.5) is a serine protease which is a 110-120 kDa transmembrane glycoprotein and cleaves N-terminal dipeptides with proline in the penultimate position (1, 2). Although the enzyme has been found to be widely distributed in mammalian tissues, its physiological role is not yet fully understood. In previous studies, we reported that serum enzyme activity was decreased in oral cancer patients and changed dynamically, reflecting clinical status during cancer therapies (3). Also, our study using the well-established hamster buccal pouch carcinogenesis model revealed that the

decrease in serum DPP IV activity occurred from the early stages, such as carcinoma *in situ* or early invasive carcinoma (4). Based on these findings, it was suggested that serum DPP IV activity might be suitable as a diagnostic or prognostic marker of malignant tumors. However, the precise mechanism of 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 plays a role in the process of proliferation of T lymphocytes (5). In the former paper (6), we reported that the number of peripheral

blood T lymphocytes was decreased and the amount of DPP IV in their plasma membranes was reduced in oral cancer patients as compared with those in healthy subjects, suggesting that suppression of DPP IV in peripheral blood T lymphocytes may be responsible for the decrease of serum DPP IV activity in cancer patients.

In this study, therefore, we further analyzed the expression of DPP IV in cultured peripheral blood T lymphocytes of oral cancer patients and the DPP IV activity in culture medium with or without mitogen stimulation, and compared them with those of healthy subjects.

Material and methods

Blood samples

Heparinized blood samples were collected from 11 newly diagnosed and untreated oral cancer patients (male:female=6:5, age 25–84 yr, average 63.7 yr) and 13 healthy subjects (male:female=7:6, age 27–79 yr, average 53.8 yr). The oral cancer patients were all diagnosed histologically as having squamous cell carcinoma and were composed of two people in Stage I, three in Stage II, four in Stage III and two in Stage IV, according to the TNM classification of the International Union Against Cancer (UICC) system (1987). Peripheral blood lymphocytes were isolated from peripheral blood by density gradient centrifugation on Lymphoprep™ (Nycomed Pharma AS, Oslo, Norway) (6). Cell viability, as determined by the trypan blue dye exclusion test, was always greater than 95%.

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, as previously described (6). Briefly, the reaction mixture containing 150 mM glycine-NaOH buffer (pH 8.7), substrate and enzyme sample was incubated at 37°C for 30 min. After termination with 1M acetate buffer (pH 4.0), the fluorescence intensity of 7-amino-4-methylcoumarine liberated was measured at 460 nm with excitation at 380 nm by a spectrofluorophotometer. The DPP IV activities in culture medium and lymphocyte extract were expressed in IU (international unit)/liter of medium and nmole/min/mg protein, respectively (6).

Cell culture and lymphocyte proliferation assay

Peripheral blood lymphocytes were cultured at 1×10^6 cells/ml in 24-well flat-bottomed microplates (Corning, Corning, NY, USA) containing RPMI 1640 (Sigma, St. Louis, MO, USA) supplemented with 10% heat-inactivated (56°C for 30 min) fetal bovine serum (Filtron Pty Ltd, Dooklyn, Australia), and 5 μ M 2-mercaptoethanol, 50 U/ml penicillin G, 50 μ g/ml streptomycin sulfate. Culture was carried out in a humidified atmosphere of 5% CO₂ in air at 37°C for 7 days. Lymphocytes were stimulated with mitogens, phytohemagglutinin (PHA-P; 10 μ g/ml: Difco, MI, USA), concanavalin A (Con A; 10 μ g/ml: Sigma) and/or T cell growth factor, interleukin-2 (IL-2; 10 BRMP units/ml: Collaborative Biochemical Products, MA, USA); all cultures were performed in triplicate. Cultured cell viability was

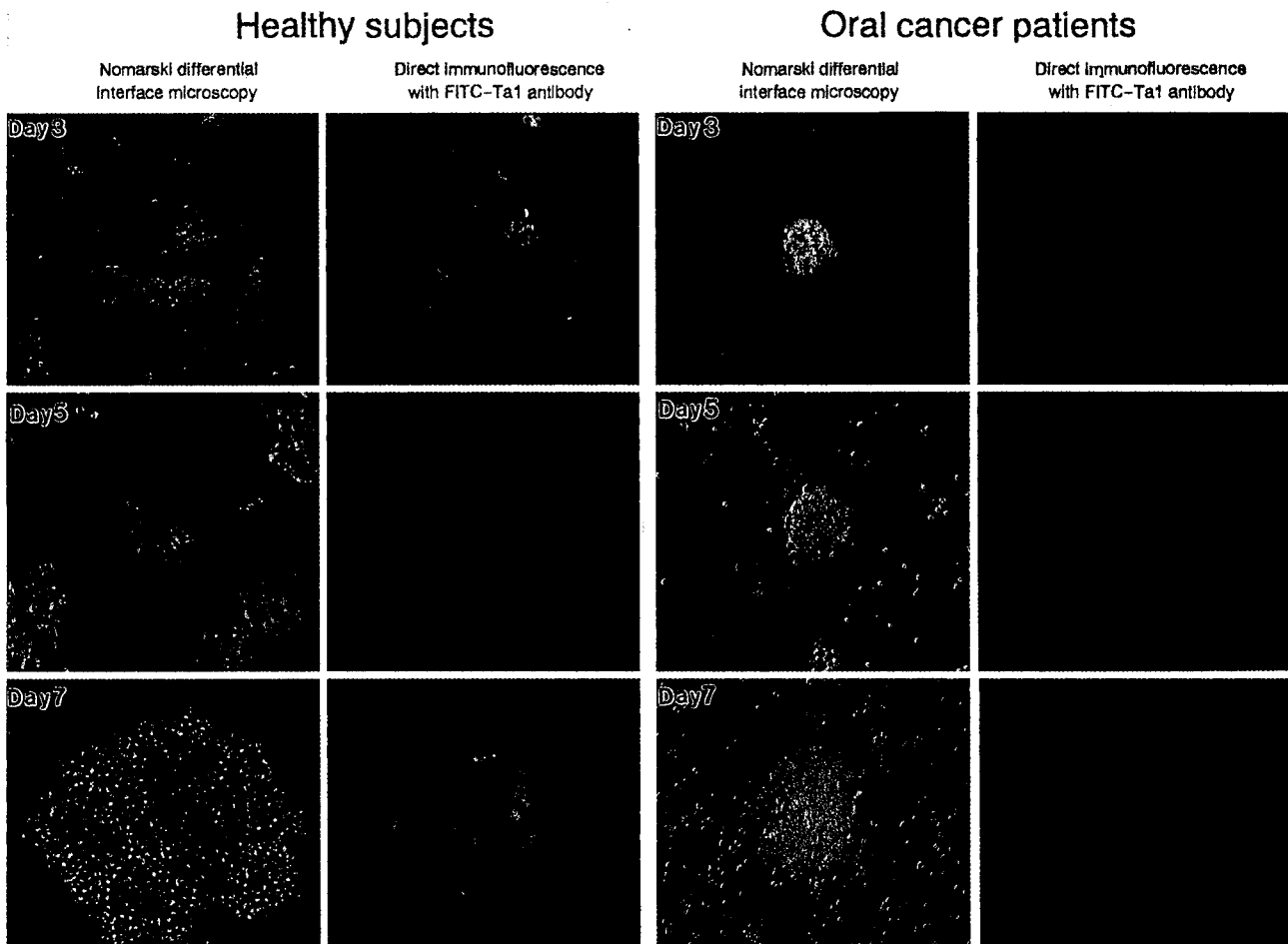


Fig. 1. Expression of CD26 in cultured peripheral blood lymphocytes of oral cancer patients and healthy subjects. Lymphocytes were cultured with 10 μ g/ml of PHA for 3, 5 and 7 days. Direct immunofluorescence staining was performed with FITC-conjugated anti-human Ta1 mouse monoclonal antibody. Note that patch and cap formation are observed in lymphocytes from healthy subjects, but not in those from cancer patients. (magnification: $\times 150$)

assayed by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-tetrazolium (MTS) method. Briefly, 100 μ l of the MTS/phenazine methosulfate (PMS) solution [mixed one-volume of PMS (0.92 mg/ml)-phosphate buffered saline (PBS) stock solution with 20-volumes of MTS (2 mg/ml)-PBS stock solution] was immediately added to each well and cultured for an additional 4 h; the absorbance at 490 nm was then measured using a microplate reader (Model 3550-UV:Bio-Rad, Tokyo, Japan). The absorbance in MTS assay and viable cell number by trypan blue dye exclusion test had previously been shown to demonstrate a linear correlation in the culture conditions up to 4×10^6 cells/ml and the correlation coefficient was 0.98 ($P < 0.05$).

Preparation of cultured lymphocyte extract

The cultured lymphocytes were solubilized by suspending a cell pellet in 0.1 M Tris-HCl (pH 7.4) buffer containing 1% Triton X-100. The lysate was then centrifuged at $12,000 \times g$ for 5 min at 4°C and the supernatant was used as the cell extract.

Direct immunofluorescence for CD26 in cultured lymphocytes

Five microliters of fluorescein isothiocyanate (FITC)-conjugated anti-human Ta1 mouse monoclonal antibody (Coulter Immunology, Hialeah, FL, USA) were added to 200 μ l of culture medium containing activated lymphocytes and then incubated at 4°C for 30 min. After washing three times with PBS, FITC-labeled lymphocytes were resuspended in Fluor-Save™ (Dakopatts a/s, Denmark) and examined by a Nomarski differential interface microscope (BX50-34-DIC: Olympus, Tokyo, Japan) and a fluorescence microscope (BX50-34-FLA1: Olympus, Tokyo, Japan).

Protein measurement

Protein concentrations were measured by a modified Lowry method (8) with the use of bovine serum albumin (BSA) as a standard.

Detection of CD26 antigen by Western immunoblotting

Ten micrograms of cultured lymphocyte extract per lane were submitted to SDS-

polyacrylamide gel electrophoresis (PAGE) in 7.5% acrylamide resolving gels, as previously described (6). Briefly, the electrophoretic transfer of proteins from SDS-PAGE to polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore Corporation, Bedford, MA, USA) was performed. After blocking with BSA, the membrane was incubated with anti-human Ta1 mouse monoclonal antibody for 2 h and then with biotinylated anti-mouse IgG rabbit antiserum (Tago Immunologicals, Burlingame, CA, USA) for 1 h at room temperature. For the color reaction, the membrane was incubated with alkaline phosphatase-labeled StreptABComplex (Dakopatts a/s) for 20 min at room temperature and the protein bands were visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma).

Statistical analysis

Statistical analysis was performed by using the Mann-Whitney U-test.

Results

Expression of CD26 (DPP IV) in cultured peripheral blood lymphocytes

When peripheral blood lymphocytes from 11 cancer patients or from 13 healthy subjects were cultured in the presence of PHA, Con A and/or IL-2,

activated T lymphocytes formed clumps. A large number of T cell clumps were observed in the lymphocyte culture of healthy subjects on day 3, and the size of the clumps increased from day 5 to 7 after cultivation. On the other hand, few T cell clumps were observed in samples from oral cancer patients even on day 5, and the clumps were smaller than those in healthy subjects. During this culture period, expression of CD26 was examined by direct immunofluorescence staining. The CD26 antigen was more strongly expressed in the T cells of healthy subjects than in those of the oral cancer patients. Patching and capping formation were frequently observed in the healthy subjects but not in the cancer patients (Fig. 1).

T cell proliferation and DPP IV activity

In lymphocyte cultures with growth medium containing PHA, Con A and/or IL-2, DPP IV activity increased markedly in the lymphocytes and culture medium in the healthy subjects but only slightly in those of the cancer patients. Although the number of lymphocytes on day 7 in the healthy subjects was increased 3.4-, 3.4- and 2.2-fold compared to the number obtained in the absence of stimulation with PHA, Con A and IL-2, respectively, the number of lymphocytes in the

Table 1. DPP IV activities in cultured lymphocyte extracts of oral cancer patients and healthy subjects

Stimulants	nmole/min/mg protein (mean \pm SD)		P-value
	Healthy subjects	Cancer patients	
Without stimulants	3.29 \pm 0.47 (1)*	2.87 \pm 0.85 (1)	N.S.
Con A	13.37 \pm 2.36 (4.1)	5.50 \pm 1.27 (1.9)	<0.001
PHA	14.40 \pm 3.41 (4.4)	5.34 \pm 1.14 (1.9)	<0.0001
IL-2	6.73 \pm 1.55 (2.1)	4.54 \pm 1.09 (1.6)	<0.01
Con A+IL-2	15.75 \pm 2.29 (4.8)	5.40 \pm 1.01 (1.9)	<0.0001
PHA+IL-2	17.52 \pm 4.33 (5.5)	6.38 \pm 1.31 (2.2)	<0.0001

*: Relative ratio, N.S.: not significant

Table 2. DPP IV activities in lymphocyte culture media of oral cancer patients and healthy subjects

Stimulants	IU/l media (mean \pm SD)		P-value
	Healthy subjects	Cancer patients	
Without stimulants	1.49 \pm 0.18 (1)*	1.45 \pm 0.09 (1)	N.S.
Con A	2.01 \pm 0.33 (1.4)	1.69 \pm 0.21 (1.2)	<0.01
PHA	2.18 \pm 0.42 (1.5)	1.80 \pm 0.33 (1.2)	<0.01
IL-2	1.77 \pm 0.38 (1.2)	1.63 \pm 0.23 (1.1)	<0.01
Con A+IL-2	2.56 \pm 0.73 (1.7)	1.82 \pm 0.30 (1.3)	<0.0001
PHA+IL-2	2.80 \pm 0.63 (1.9)	2.04 \pm 0.41 (1.4)	<0.0001

*: Relative ratio, N.S.: not significant

cancer patients was increased only 1.6-, 1.7- and 1.5-fold, respectively. The cell growth of T lymphocytes was enhanced by the addition of IL-2 with PHA or Con A. Furthermore, the DPP IV activity of healthy subjects was elevated 2.1- to 5.5-fold in lymphocyte extracts and 1.2- to 1.9-fold in culture medium concomitant with the proliferation of T lymphocytes, whereas DPP IV activity of cancer patients was elevated only 1.6- to 2.2- fold and 1.1- to 1.4- fold, respectively (Tables 1 and 2). Although T cell proliferation and DPP IV activities in cell extract and culture medium were significantly increased in healthy subjects as compared to cancer patients, there was no significant difference between the clinical stages of cancer patients, so far as could be determined from 11 patients. Figure 2 represents a typical example of the T cell growth curve and DPP IV activities in cell extracts and culture media in the absence and presence of PHA.

Detection of CD26 (DPP IV) protein in cultured lymphocytes

To measure the amount of CD26 (DPP IV) protein in cultured lymphocytes, SDS-PAGE and Western immunoblot analysis with Ta1 mouse monoclonal antibody were performed. When lymphocytes were cultured with a stimulant-free medium for 7 days, CD26 (DPP IV) protein was not detected in any sample. However, it was detected in the cell extracts of healthy subjects on day 3, and the expression was enhanced on day 7 after cultivation with PHA stimulation. On the other hand, the protein band was not shown clearly in the cell extracts of oral cancer patients on day 3 and was only faintly detected on day 7. The amount of CD26 protein in cultured lymphocytes was markedly less in oral cancer patients (data not shown).

Discussion

The cytochemical detection of DPP IV in human peripheral blood lymphocytes was first reported by LOJDA (9). The precise function of DPP IV in human lymphocytes is still unknown, but DPP IV is largely confined to T lymphocytes and is implicated in lymphocyte proliferation. At the Fourth International Workshop on Human Leucocyte Differentiation Antigens (1989), DPP IV was defined as CD26, one of the leucocyte surface

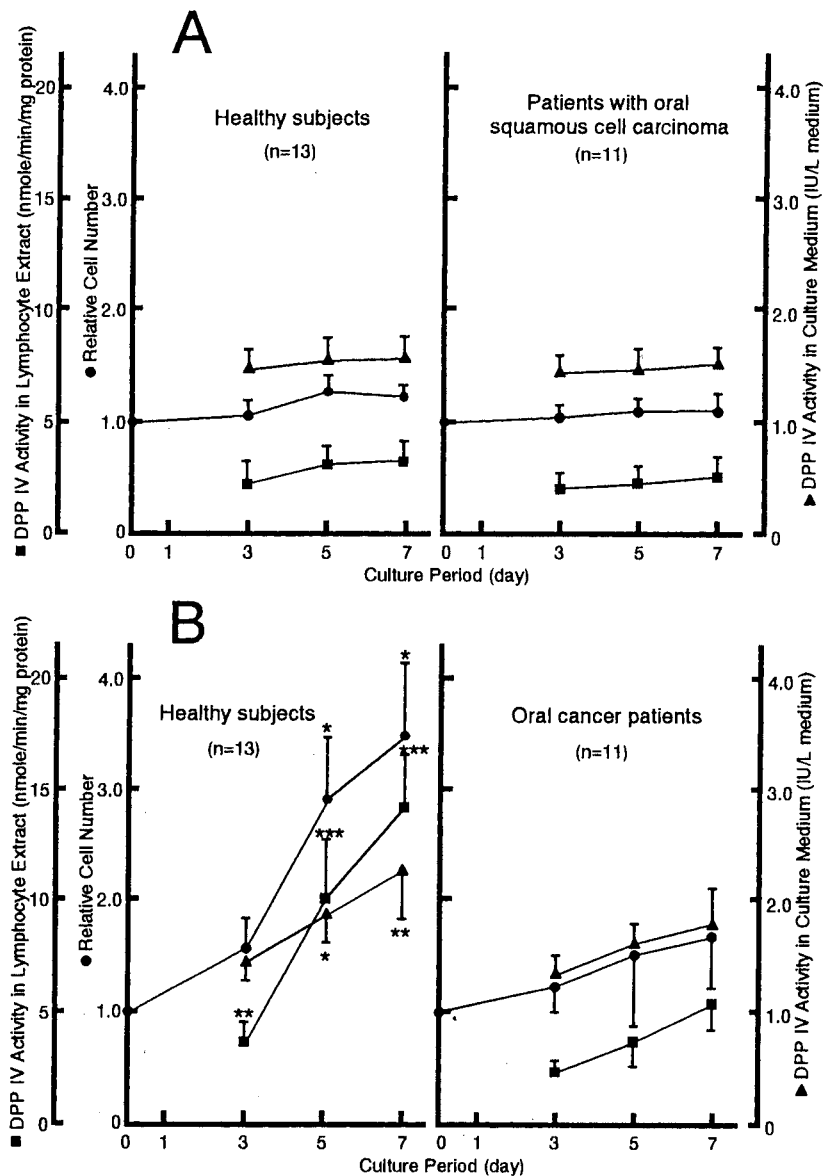


Fig. 2. Relative cell number, DPP IV activity of lymphocyte extract, and DPP IV activity of culture medium in lymphocyte cultures of oral cancer patients and healthy subjects. Lymphocytes were cultured without PHA (A) or with 10 µg/ml of PHA (B) for 7 days. Values are significantly decreased in patients with oral cancer (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$) as compared to healthy subjects.

markers involved in the activation of T lymphocytes (5). SCHÖN *et al.* (10) reported that inhibitors and antibodies against DPP IV suppressed lymphocyte proliferation induced by mitogenic lectins (PHA, Con A, pokeweed mitogen) and markedly reduced immunoglobulin production by pokeweed mitogen-stimulated lymphocytes as a consequence of impaired T cell function. KASAHARA *et al.* (11) demonstrated clinically in four patients with rheumatoid arthritis that 4 weeks of thoracic duct drainage resulted in a reduction in the peripheral blood lymphocyte count and a concomitant decrease of serum DPP IV activity.

These findings suggest that reduction in the number of lymphocytes and lymphocyte functions has an influence on the decrease in serum DPP IV activity.

Cellular immunity mediated by immunocompetent cells such as macrophages, natural killer cells, and peripheral blood T lymphocytes plays an important role in the immune surveillance that checks the development of malignant cells. On the assumption that suppression of cellular immunity allows malignant cells to proliferate *in vivo*, it is readily speculated that decrease in the number of T lymphocytes and depres-

sion of T cell function have an influence on the expression of CD26 in T lymphocytes, and thereby may bring about the reduction of serum DPP IV activity. We found in a previous study that DPP IV activity and the amount of CD26 in peripheral blood T lymphocyte plasma membranes were greatly reduced in cancer patients compared to healthy subjects (6). Therefore, it is necessary to examine how DPP IV activity in activated T lymphocyte plasma membranes is changed and whether DPP IV is released from the plasma membrane into the extracellular space.

In this study we investigated T lymphocytes obtained from oral cancer patients and healthy subjects and then cultured with PHA and Con A as T cell stimulants and with IL-2 as a T cell growth factor. In the cultured lymphocytes of healthy subjects, DPP IV activity and CD26 antigen expression increased as the response of T lymphocytes to PHA, Con A and IL-2 developed. In the cancer patients, however, the reactivity of T lymphocytes to these stimulants was diminished, and DPP IV activities in lymphocyte extracts and culture media were lower than those in healthy subjects. Furthermore, the amount of DPP IV enzyme protein in activated lymphocytes was markedly reduced in cancer patients. Since it is known that the antigens located on the cell surface of both normal and cancer cells are released into serum or medium by shedding (12), it is considered that the reduction of CD26 expression in T lymphocytes is important in attempts to explain the mechanism of decrease of serum DPP IV activity in oral cancer patients. So far as has been examined with 11 patients, we could not find significant differences between clinical stages of cancer patients and the extent of the reduction of CD26 expression in T lymphocytes; nevertheless, DPP IV activity and CD26 expression in peripheral

blood T lymphocytes, as well as serum DPP IV activity, could be valuable aids for the diagnosis and prognosis of cancer patients.

At present, the precise cause of T cell suppression in cancer patients is unclear. Recently, it was reported that TGF- β suppresses the functions of CD4- and CD8-positive T cells and lymphokine-activated killer (LAK) cells, resulting in immunosuppression in the tumor-bearing state (13, 14). It would therefore be of interest to elucidate whether the cytokines produced by tumor cells are the key factor in the induction of the decrease of serum DPP IV activity in cancer patients.

Acknowledgements – The authors thank Drs. M. HARADA and M. MOGI, Department of Oral Biochemistry, Matsumoto Dental College, Nagano, Japan for their valuable suggestions. This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan to T. U. (No. 07771998).

References

1. HOPUSU-HAVU VK, GLENNER GG. A new dipeptide naphthylamidase hydrolyzing glycyL-prolyl- β -naphthylamide. *Histochemie* 1966; **7**: 197–201.
2. McDONALD JK, CALLAHAN PX, ELLIS S, SMITH RE. Polypeptide degradation by dipeptidyl aminopeptidase I (cathepsin C) and related peptidases. In: BARRET AJ, DINGLE JT, eds. *Tissue proteinases*. Amsterdam: Elsevier/North Holland, 1971: 69–107.
3. URADE M, KOMATSU M, YAMAOKA M, *et al.* Serum dipeptidyl peptidase activities as a possible marker of oral cancer. *Cancer* 1989; **64**: 1274–80.
4. URADE M, UEMATSU T, MIMA T, OGURA T, MATSUYA T. Serum dipeptidyl peptidase (DPP) IV activity in hamster buccal pouch carcinogenesis with 9, 10-dimethyl-1, 2-benzanthracene. *J Oral Pathol Med* 1992; **21**: 109–12.
5. KNAPP W, RIEBER P, DORKEN B, SCHMIDT RE, STEIN H, BORNE AEG KRVD. Towards a better definition of human leucocyte surface molecules. *Immunol Today* 1989; **10**: 253–8.
6. UEMATSU T, URADE M, YAMAOKA M, YOSHIOKA W. Reduced expression of dipeptidyl peptidase (DPP) IV in peripheral blood T lymphocytes of oral cancer patients. *J Oral Pathol Med* 1996; **25**: 507–12.
7. KATO T, NAGATSU T, KIMURA T, SAKAKIBARA S. Fluorescence assay of X-prolyl dipeptidyl-aminopeptidase activity with a new fluorogenic substrate. *Biochem Med* 1978; **19**: 351–9.
8. SIMPSON IA, SONNE O. A simple, rapid, and sensitive method for measuring protein concentration in subcellular membrane fractions prepared by sucrose density ultracentrifugation. *Anal Biochem* 1982; **119**: 424–7.
9. LOJDA Z. Studies on glycyLproline naphthylamidase. *Histochemistry J* 1977; **54**: 299–309.
10. SCHÖN E, JAHN S, KIESSIG ST, *et al.* The role of dipeptidyl peptidase IV in human T lymphocyte activation. Inhibitors and antibodies against dipeptidyl peptidase IV suppress lymphocyte proliferation and immunoglobulin synthesis *in vitro*. *Eur J Immunol* 1987; **17**: 1821–6.
11. KASAHARA Y, LEROUX-ROELS G, NAKAMURA R, CHISARI F. GlycyLprolyl-diaminopeptidase in human leukocytes: selective occurrence in T lymphocytes and influence on the total serum enzyme activity. *Clin Chim Acta* 1984; **139**: 295–302.
12. BLACK PH. Shedding from the cell surface of normal and cancer cells. *Adv Cancer Res* 1980; **32**: 75–199.
13. TADA T, OHZEKI S, UTSUMI K, *et al.* Transforming growth factor- β -induced inhibition of T cell function: susceptibility difference in T cells of various phenotypes and functions and its relevance to immunosuppression in the tumor-bearing state. *J Immunol* 1991; **146**: 1077–82.
14. SMYTH MJ, STROBLE SL, YOUNG HA, ORTALDO JR, OCHOA AC. Regulation of lymphokine-activated killer activity and pore-forming protein gene expression in human peripheral blood CD8⁺ T lymphocytes: inhibition by transforming growth factor- β . *J Immunol* 1991; **146**: 3289–97.