ANTICANCER RESEARCH *24*: 619-624 (2004)

Effects of Oral Squamous Cell Carcinoma-derived TGF-β1 on CD26/DPPIV Expression in T Cells

TAKASHI UEMATSU[1,2], HITOSHI TANAKA[1], MINORU YAMAOKA[1] and KIYOFUMI FURUSAWA[1,2]

[1]Department of Oral and Maxillofacial Surgery and [2]Institute for Oral Science, Matsumoto Dental University School of Dentistry, Nagano, Japan

Abstract. *Background: Enzymatic activity levels of serum CD26/ dipeptidylpeptidase (DPP) IV in oral cancer patients are lower than those in healthy subjects. However, the mechanism for this decrease is not yet fully understood. Materials and Methods: The influence of malignant cell-derived cytokines on cell surface CD26/DPP IV expression in human T cells was analyzed using an oral squamous cell carcinoma (SCC) cell line, KB, and peripheral blood T cells. Results: Tumor growth factor (TGF)-β_1 in KB-conditioned medium (KBCM) down-regulated CD26/DPPIV expression in T cells, which was responsible for the decreased DPPIV activities in the cultured supernatant. Expression $p27^{kip}$ in T cells was maintained in addition to G1 arrest when cultured with KBCM but was abolished by inclusion of anti-TGF-β_1 antibody. Conclusion: SCC-derived TGF-β_1 down-regulates CD26/ DPPIV expression in T cells, resulting in decreased serum CD26/DPPIV activity in oral cancer patients.*

Dipeptidylpeptidase IV (DPP IV, EC 3.4.14.5) is an exopeptidase that preferentially catalyzes the release of N-terminal dipeptides from polypeptides with proline in the penultimate position (1). This enzyme was found to be identical to cell surface CD26 antigen (CD26 / DPP IV), which is expressed in T cells (2). CD26/DPPIV activity has also been observed in human sera and was found to originate from T cells (3). Previous studies have demonstrated that serum-soluble CD26/DPPIV (sCD26) or enzymatic activity levels in oral cancer patients were lower than those in their counterparts (4) and that this is indicative of clinical status (5). The well-established hamster buccal pouch carcinogenesis model revealed that decreases in serum DPPIV activity occurred in carcinoma *in situ* or with early invasive carcinoma (6). Furthermore, it was demonstrated that DPPIV activity in T cells was correlated with that in T cell culture supernatant in *in vitro* studies (7). These reports suggest that sCD26 activity in human sera is affected by the expression of CD26/DPPIV in T cells.

Therefore, in the present study, we analyzed the malignant cell-derived soluble factors that influence the CD26/DPPIV expression of T cells using head and neck carcinoma cell lines and human peripheral blood T cells.

Correspondence to: Takashi Uematsu, Department of Oral and Maxillofacial Surgery, Matsumoto Dental University School of Dentistry, 1780 Gobara Hirooka, Shiojiri, Nagano 399-0781, Japan. Tel: +81-263-52-3100, Fax: +81-263-51-2066, e-mail: uematsu@po.mdu.ac.jp

Key Words: Tumor marker, squamous cell carcinoma, CD26, TGF-β_1, $p27^{kip}$.

Materials and Methods

Cell culture and assay for DPPIV activity. Peripheral blood T cells were isolated and T cell extracts were prepared as described previously (7). T cells were cultured in serum-free medium (SFM 101; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). Proliferation of T cells was studied after activation by phytohemagglutinin (PHA-P; Difco, MI, USA) at a concentration of 10 μg/ml. Human squamous cell carcinoma cell lines, KB (8), Hepd (9) and human salivary gland adenocarcinoma cell lines, HSG (10) and HSY (11), were maintained as described previously, and were then adapted to SFM-101 medium by stepwise sequential exposure to SFM-101 medium. Using 24-well plates and Membrane Culture Inserts™ (Iwaki Glass Co. Ltd, Osaka, Japan), $1x10^5$ carcinoma cells and the same number of T cells were cultured in the lower and upper chambers, respectively. Culture was carried out in a humidified atmosphere of 5% CO_2 in air at 37°C.

Fractionation of KBCM proteins and assay for cytokines. The 8-30-kDa KBCM fraction was concentrated using Centricon Plus-20 (Millipore, Bedford, MA, USA), subjected to fractionation by heparin affinity chromatography and then eluted by NaCl into 100 fractions using the ÄKTA FPLC system (Amersham Pharmacia Biotech, Buckinghamshire, UK). Cytokines were assayed using ELISA kits (R & D systems, Minneapolis, MN, USA) according to the manufacturer's instructions and as described by Danielpour (12).

Flow cytometry. The following fluorochrome-conjugated antibodies were used: phycoerythrin (PE) conjugated anti-CD3 (clone M2AB, Exalpha Biologicals, San Jose, CA, USA), anti-CD4 (clone 7E14, Becton Dickinson, San Jose, CA, USA) and anti-CD8 (clone 7D8, Becton Dickinson), fluorescein (FITC) conjugated anti-CD26

Table I. *T cell number, DPPIV activity of T cell extract and DPPIV activity in T cell culture supernatant in simultaneous culture of T cells with head and neck carcinoma cells.*

Cell lines[1]	T cell number (x10^4) (mean±S.D.)	DPP IV activity (mean±S.D.)	
		T cell extract (nmol/min/mg protein)	Culture supernatant (IU/l)
Control[2]	31.7±1.0	11.19±1.10	2.15±0.13
KB	19.2±0.8[6]	5.13±0.21[6]	1.63±0.05[6]
Hepd	23.3±0.4[5]	9.87±0.37[3]	1.86±0.02[4]
HSY	28.4±0.3[5]	9.83±0.63[3]	1.87±0.03[4]
HSG	29.2±0.3[3]	8.67±0.15[4]	1.83±0.02[4]

[1]T cells (1 x 10^5 cells) were simultaneously cultured with a carcinoma cell line (1 x 10^5 cells) in SFM101 with PHA at a concentration of 10 μg/ml for 5 days (n=6).
[2]T cells were cultured without a carcinoma cell line.
[3-6]Significant difference from control: [3]$p<0.05$, [4]$p<0.01$, [5]$p<0.001$, [6]$p<0.0001$

(Clone EUG-6, Coulter Immunology, Hialeah, FL, USA) and IgG1Î isotype control antibody (Becton Dickinson). Cells were analyzed for DNA content using propidium iodide (PI) solution (50 μg/ml in PBS, pH 7.4) containing Triton X-100 (0.1% v/v) and DNase-free RNase (1 μg/ml). The proportion of G0/G1-phase cells was estimated as a percentage of total cells (5x10^3 cells).

Western blot analysis. Expression of p27^{kip} and CD26/DPPIV in T cells was analyzed by Western blot using anti-p27^{kip} antibody (Upstate Biotechnology, Waltham, MA, USA) or anti-CD26 antibody (Santa Cruz Biotechnology, Inc., CA, USA). Blots were subsequently incubated with an HRP-conjugated goat anti-rabbit antibody and proteins were visualized with the ECL Western blotting detection system (Amersham Pharmacia Biotech) using a VersaDog Model 5000 (Bio-Rad, Hercules, CA, USA).

Statistics. Continuous data were compared using the Student's *t*-test if the distribution of samples was normal, or the Mann-Whitney *U*-test if sample distribution was asymmetrical.

Results

Influence of malignant cell-derived factors on T cells. In the co-culture of T cells and KB cells, DPPIV activity decreased markedly in the T cell extracts and culture supernatant, when compared to other co-cultures (Table I). In order to clarify the approximate molecular weight of the T cell suppressor protein derived from malignant cells, KB cells which markedly suppressed T cell proliferation and DPPIV activity in T cells and culture supernatant were subjected to the following experiments. KB-conditioned medium (KBCM) was fractionated using Centricon Plus-20 into 6

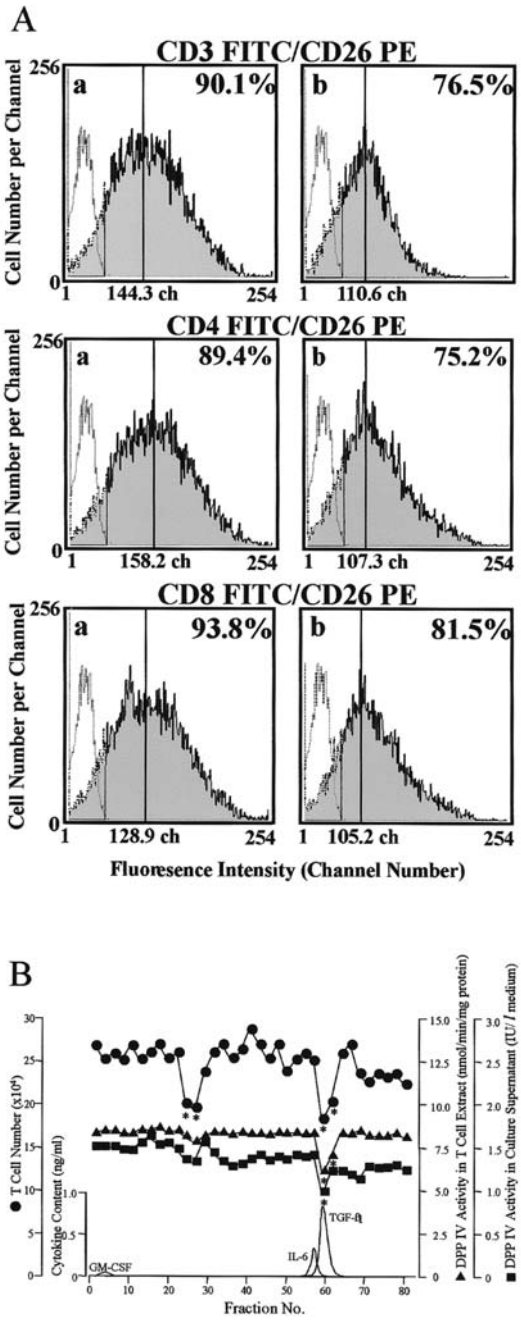

Figure 1. *Influence of 8-30 kDa KBCM proteins on CD26/DPPIV expression. (A) Normal human T cells were cultured in growth medium that did not contain (a) or did contain (b) 30 μg of protein extracted from the 8-30-kDa KBCM fraction. Fluorescence histograms are shown: gray histograms were produced using antibodies specific to cell surface antigens; white histograms were produced using a control labeled with the mouse IgG1 isotype antibody. The rates shown are ratios of CD26$^+$ cell number to total T cell subpopulation. (B) 8-30-kDa KBCM fraction was subjected to fractionation by heparin affinity chromatography and eluted with NaCl into 100 fractions. Cytokines present in the fractions were determined by ELISA. T cells were cultured with each protein fraction. T cell number (closed circle), DPPIV activity of T cell extract (closed square) and DPPIV activity of the culture medium (closed triangle) were assayed. Results shown are means of triplicate assays. Values significantly different from those in the control: *$p<0.05$.*

fractions, < 5 kDa, 5-8 kDa, 8-30 kDa, 30-50 kDa, 50-100 kDa, > 100 kDa by nominal molecular weight limits in kiloDaltons (NMWL). After 5 days of T cell cultivation in growth medium that contained 30 μg of the 8-30-kDa fraction, a 28% decrease in T cell growth was observed, the CD26/DPPIV activity of the culture supernatant had decreased by 22% and the DPPIV activity of the T cell extract had decreased by 8%. In flow cytometric analysis, the number of $CD3^+/CD26^+$, $CD4^+/CD26^+$ and $CD8^+/CD26^+$ cells was 13.6%, 14.2% and 12.3% lower, respectively, than for their respective counterparts. Furthermore, mean values of CD26-fluorescence intensity in histograms shifted from 144.3 ch to 110.6 ch in the $CD3^+/26^+$ cells, from 158.2 ch to 107.3 ch in the $CD4^+/CD26^+$ cells and from 128.9 ch to 105.2 ch in the $CD8^+/CD26^+$ cells, indicating that proteins in the 8-30-kDa fraction were suppressing CD26/DPPIV expression in T cells, particularly in $CD4^+$ T cells (Figure 1A).

Detection of KB-derived cytokines. In order to clarify the factor that was suppressing CD26/DPPIV expression, a fraction of the 8-30-kDa KBCM was subjected to fractionation by heparin affinity chromatography and was eluted with NaCl into 100 fractions. In T cell cultivation with 10 μg of each fraction protein, fractions #58-64 significantly influenced T cell growth and DPPIV activity in cell extracts and culture supernatant. Moreover, the T cell number was significantly reduced by addition of fractions #25 and 26 to the culture supernatant while DPPIV activity was slightly but not significantly suppressed. When the cytokines in each fraction were screened and quantified by ELISA, GM-CSF, IL-6 and TGF-β_1 were detected in fractions #1-7, #55-59 and #58-64, respectively. Thus, it was suggested that the decrease in T cell growth and DPPIV activity in the T cell extract and culture supernatant was due to TGF-β_1. However, no known cytokines were detected in fractions #25 and 26, suggesting that these fractions contained unknown inhibitory proteins (Figure 1B).

Neutralizing assay. After addition of anti-TGF-β_1, anti-GM-CSF or anti-IL-6 antibodies to 30% KBCM, T cells were cultured with the 30% KBCM and T cell growth as well as DPPIV activity in the T cell extract and culture supernatant were measured. Following neutralization with 1 μg/ml of anti-TGF-β_1, 62.4% of T cell growth, and 87.8% and 64.7% of the DPPIV activity in the T cell extract and culture supernatant, respectively, were recovered after cultivation for 5 days. However, 1 μg/ml of anti-GM-CSF and -IL-6 antibodies did not substantially reverse the inhibitory effects in 30% KBCM (Figure 2A). The concentrations of IL-2, IL-4 and IFN-γ in the culture supernatant increased over time when cultured with PHA, but decreased when cultured with

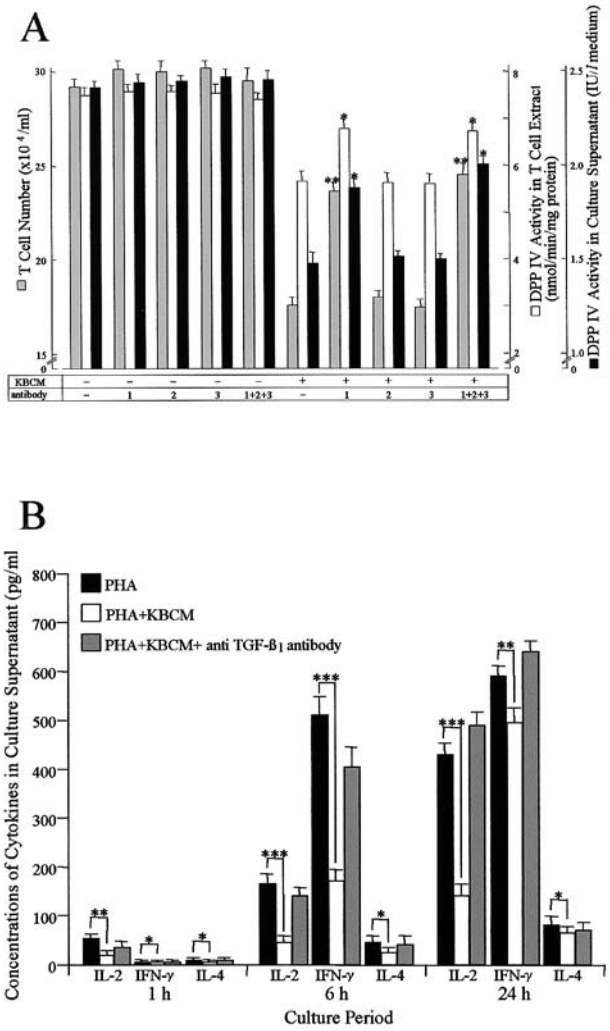

Figure 2. *Neutralizing assay for cytokines in KBCM. (A) Normal human peripheral blood T cells were cultured in 30% KBCM with or without 1 μg/ml of cytokine-neutralizing antibodies for 5 days. T cell number (gray bar), DPPIV activity of T cell extract (clear bar), and DPPIV activity of culture supernatant (solid bar) were assayed. Bars indicate SD. KBCM (-), not supplemented with 30% KBCM; KBCM (+), supplemented with 30% KBCM; antibody (-), no addition of antibody; 1, addition of anti-TGF-β_1 antibody (1 μg/ml); 2, addition of anti-GM-CSF antibody (1 μg/ml); 3, addition of anti-IL-6 antibody (1 μg/ml). Values significantly different from those in KBCM (+) and antibody (-). (B) Detection of Th1 and Th2 type cytokines. IL-2, IL-4 and IFN-γ in culture supernatant were measured by ELISA after T cells were cultured for 1, 6 or 24 h in culture medium containing 30% KBCM with or without neutralizing antibodies: Values significantly different from those in the control: *p<0.05, **p<0.01, ***p<0.001.*

PHA and 30% KBCM. Levels of IL-2 and IFN-γ decreased substantially when compared with those of IL-4, which was neutralized by anti-TGF-β_1 antibody, indicating that the function of Th1-type T cells are more readily affected by KB-derived TGF-β_1 (Figure 2B).

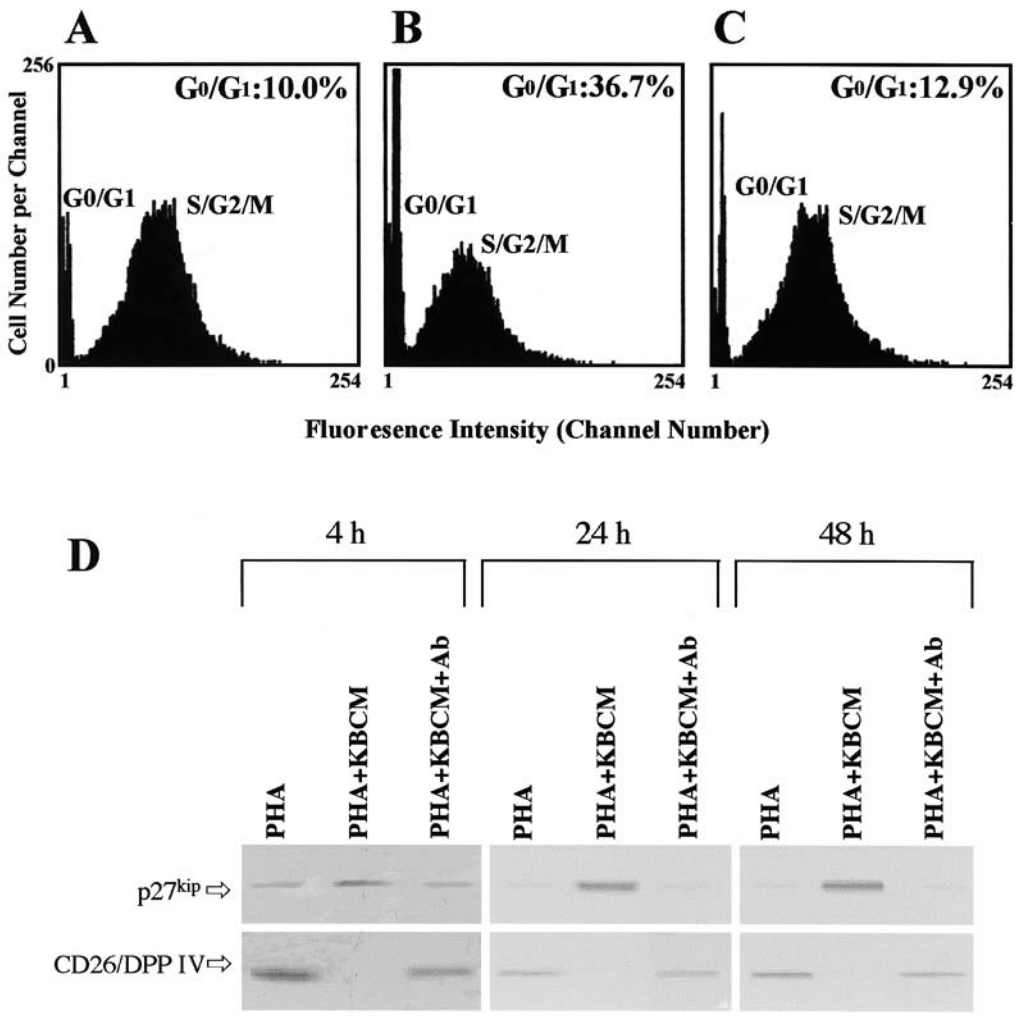

Figure 3. *Cell cycle arrest, p27^{kip} and CD26/DPPIV expression in T cells. A-C: T cells were cultured in SFM 101 containing 10 μg/ml PHA (A), with 30% KBCM (B), or with 30% KBCM and anti-TGF-β_1 antibody (1 μg/ml) (C). After 48 h of cultivation, T cells were stained with PI and analyzed by flow cytometry. The rates shown are ratios of the G0/G1 cell number to the total cell number. D: Expression of p27^{kip} and CD26/DPPIV in cultured human peripheral blood T cells. After T cell cultivation in SFM 101 medium containing PHA with or without KBCM or anti-TGF-β_1 antibody for 6, 24 or 48 h, 10 μg of T cell extract from each culture was subjected to SDS-PAGE and probed using anti-p27^{kip} and anti-CD26 antibodies. PHA, T cell cultivation with 1 μg/ml of PHA; PHA+KBCM, T cell cultivation with 1 μg/ml of PHA and 30% KBCM; PHA+KBCM+Ab, T cell cultivation with 1 μg/ml of PHA, 30% KBCM and 1 μg/ml of anti-TGF-β_1 antibody.*

Influence of KBCM on cell cycle progression of T cells. In an attempt to elucidate the blocking progression of PHA-stimulated T cells through the cell cycle, PI staining of T cells was performed and histograms were compared after 48 h of cultivation. Representative results presented in Figures 3 A-C suggest that the proportion of cells within the G0/G1-phase in the presence of 30% KBCM (36.7%, Figure 3B) was higher than that in the absence of 30% KBCM (10.0%, Figure 3A). Furthermore, the proportion of cells within the G0/G1-phase was reversed by addition of 1 μg/ml of anti-TGF-β_1 antibody (12.9%, Figure 3C). In Western blot analysis, the level of p27^{kip} expression in T cells diminished on cultivation with PHA for 24 h. Concomitant with the decreased expression of CD26/DPPIV, however, the p27^{kip} expression levels remained constant in T cell extract cultured with PHA and 30% KBCM for 48 h. Moreover, the p27^{kip} expression in T cells cultured with PHA and 30% KBCM was abolished by incubation with anti-TGF-β_1 antibody, resulting in an increase in CD26/DPPIV expression by T cells (Fig. 3D). KB-derived TGF-β_1 was thus shown to down-regulate CD26/DPPIV expression by maintaining levels of p27^{kip} expression in T cells.

Discussion

The present study found that KB-derived mature TGF-β_1 suppressed CD26/DPPIV expression in cultured human T cells. This down-regulation of CD26/DPPIV expression was responsible for the decrease in DPPIV activity observed in the culture supernatant. This was due to expression of the CDK2 inhibitor $p27^{kip}$, which is associated with cell cycle arrest in the late G1-phase in T cells. KB-derived mature TGF-β_1 inhibited T cell proliferation, particularly in $CD4^+$ cells, and suppressed production of IL-2 and IFN-γ. The CD26/DPPIV suppression seen in the present study demonstrates that oral squamous carcinoma cell-derived soluble factors inhibit cell proliferation, cell activation and immune function of T cells and suggests that this results in decreased serum CD26/DPPIV activity in oral cancer patients.

Previous studies have suggested that several parameters in sera are potentially useful as prognostic makers for head and neck carcinomas (13, 14). However, a practical tumor marker in sera that reflects immune function in patients with oral cancer under the influence of malignant cells has not yet been reported. TGF-β_1 is produced by oral squamous cell carcinomas as well as keratinocytes (15), suggesting that oral squamous cell carcinoma-derived TGF-β_1 could biologically influence healthy cells and tissues, particularly T cell activation and expression of CD26/DPPIV. TGF-β_1 potently induces G1-phase cell cycle arrest by inhibiting cyclin D- and cyclin E-associated kinase complexes. Down-regulation of kinase activity is mediated by induction of cyclin-dependent kinase inhibitor p15 (Ink4b), which blocks CDK4 and CDK6 kinases and leads to binding of $p27^{kip}$ to the CDK2-cyclin E complex (16). Therefore, mature TGF-β_1 produced by cancer cells down-regulates CD26/DPPIV expression in T cells concomitant with suppression of T cell growth by maintaining $p27^{kip}$ expression, which leads to cell cycle arrest in G1, resulting in decreased serum CD26/DPPIV activity in oral cancer patients.

In a previous study, decreased CD26 expression in human peripheral blood T cells was detected in $CD4^+$ T cells when compared with $CD8^+$ T cells in oral cancer patients (17). CD26 antigen is involved in CD3- and CD2-induced human $CD4^+$ T cell activation (18). This implies that down-regulation of CD26 expression and T cell inactivation may be involved in suppression of cellular immunity in cancer patients. In FCM analysis, DPPIV expression was suppressed in both $CD4^+$ and $CD8^+$ cells, particularly in the $CD4^+$ cells, by cultivation with KBCM. A significant decrease in active secretion was observed for the Th1-type cytokines IFN-γ and IL-2 in T cells cultivated with KBCM, while endogenous production of the Th2-type cytokine IL-4 was slightly decreased. This finding is similar to clinical observations that Th2 cells are more prevalent than Th1 cells in cancer patients (19, 20).

In conclusion, the present study demonstrated that the decrease in soluble CD26/DPPIV activity is due to malignant cell-derived TGF-β_1 and may ultimately contribute to a better understanding of the clinical applications of serum CD26/DPPIV activity as a possible diagnostic/predictive marker in oral cancer patients.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (07771998, 10470444, 12771254, 14771168).

References

1 Yaron A and Naider F: Proline-dependent structural and biological properties of peptides and proteins. Crit Rev Biochem Mol Biol *28*: 31-81, 1993.
2 Schön E, Demuth HU, Barth A and Ansorge S: Dipeptidyl peptidase IV of human lymphocytes. Evidence for specific hydrolysis of glycylproline p-nitroanilide in T-lymphocytes. Biochem J *223*: 255-258, 1984.
3 Durinx C, Lambeir AM, Bosmans E, Falmagne JB, Berghmans R, Haemers A, Scharpe S and De Meester I: Molecular characterization of dipeptidyl peptidase activity in serum: soluble CD26/dipeptidyl peptidase IV is responsible for the release of X-Pro dipeptides. Eur J Biochem *267*: 5608-5613, 2000.
4 Mogi M, Harada M, Hiraoka BY, Fukasawa K, Komatsu M and Nagatsu T: Sandwich enzyme-immunoassay for dipeptidyl aminopeptidase IV in the serum of people with oral cancer. Arch Oral Biol *31*: 505-507, 1986.
5 Urade M, Komatsu M, Yamaoka M, Fukasawa K, Harada M, Mima T and Matsuya T: Serum dipeptidyl peptidase activities as a possible marker of oral cancer. Cancer *64*: 1274-1280, 1989.
6 Urade M, Uematsu T, Mima T, Ogura T and Matsuya T: Serum dipeptidyl peptidase (DPP) IV activity in hamster buccal pouch carcinogenesis with 9,10-dimethyl-1,2-benzanthracene. J Oral Pathol Med *21*: 109-112, 1992.
7 Uematsu T, Urade M and 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 *27(3)*: 106-110, 1998.
8 Eagle H: Propagation in a medium of a human epidermoid carcinoma, strain KB. Pro Soc Exp Biol Med *89*: 362-364, 1955.
9 Urade M, Sugi M and Miyazaki T: Establishment of three bleomycin-resistant human carcinoma cell lines and their cross-resistance to other antitumor agents. Cancer *61*: 1501-1507, 1988.
10 Shirasuna K, Watatani K, Sugiyama M, Morioka S and Miyazaki T: Isolation and characterization of different clones including myoepithelial-like variants from a clonal neoplastic epithelial duct cell line of human salivary gland origin. Cancer Res *46*: 1418-1426, 1986.
11 Hayashi Y, Yanagawa T, Yoshida H, Azuma M, Nishida T, Yura Y and Sato M: Expression of vasoactive intestinal polypeptide and amylase in a human parotid gland adenocarcinoma cell line in culture. J Natl Cancer Inst *79*: 1025-37, 1987.
12 Danielpour D: Improved sandwich enzyme-linked immunosorbent assays for transforming growth factor beta 1. J Immunol Methods *158*: 17-25, 1993.

13 Brockmann JG, St Nottberg H, Glodny B, Heinecke A and Senninger NJ: CYFRA 21-1 serum analysis in patients with esophageal cancer. Clin Cancer Res *6*: 4249-4252, 2000.
14 Yamamoto N, Naraparaju VR and Urade M: Prognostic utility of serum alpha-N-acetylgalactosaminidase and immunosuppression resulted from deglycosylation of serum Gc protein in oral cancer patients. Cancer Res *57*: 295-299, 1997.
15 Game SM, Huelsen A, Patel V, Donnelly M, Yeudall WA, Stone A, Fusenig NE and Prime SS: Progressive abrogation of TGF-beta 1 and EGF growth control is associated with tumour progression in ras-transfected human keratinocytes. Int J Cancer *52*: 461-470, 1992.
16 Sandhu C, Garbe J, Bhattacharya N, Daksis J, Pan CH, Yaswen P, Koh J, Slingerland JM and Stampfer MR: Transforming growth factor beta stabilizes p15INK4B protein, increases p15INK4B-cdk4 complexes, and inhibits cyclin D1-cdk4 association in human mammary epithelial cells. Mol Cell Biol *17*: 2458-2467, 1997.
17 Uematsu T, Urade M, Yamaoka M and Yoshioka W: Reduced expression of dipeptidyl peptidase (DPP) IV in peripheral blood T lymphocytes of oral cancer patients. J Oral Pathol Med *25*: 507-512, 1996.
18 Dang NH, Torimoto Y, Deusch K, Schlossman SF and Morimoto C: Comitogenic effect of solid-phase immobilized anti-1F7 on human CD4 T cell activation *via* CD3 and CD2 pathways. J Immunol *144*: 4092-4100, 1990.
19 Agarwal A, Rani M, Saha GK, Valarmathi TM, Bahadur S, Mohanti BK and Das SN: Disregulated expression of the Th2 cytokine gene in patients with intraoral squamous cell carcinoma. Immunol Invest *32*: 17-30, 2003.
20 Pignataro L, Corsi MM, Sambataro G, Porcaro L, Tredici P and Broich G: Plasmatic cytokine network in patients with laryngeal carcinoma after surgical treatment. Anticancer Res *21*: 3621-3625, 2001.

Received September 1, 2003
Accepted December 28, 2003