**FIGURE 5.** EGR2 expression strongly induced via CD26-mediated costimulation is associated with IL-10 production. (**A** and **B**) Freshly purified CD4$^+$ T cells were stimulated with anti-CD3 alone, anti-CD3 plus anti-CD28 mAbs (25 μg/ml), or anti-CD3 plus anti-CD26 mAbs (25 μg/ml) for the indicated times. (A) mRNA expression of EGR2 (**a**), IRF4 (**b**), c-Maf (**c**), and Blimp-1 (**d**) was quantified by real-time RT-PCR. Each expression was normalized to HPRT1, and relative expression levels compared with resting CD4$^+$ T cells (0 h) are shown. Representative data of four independent donors are shown as mean ± SD of triplicate samples, comparing values in anti-CD3 plus anti-CD26 to those in anti-CD3 plus anti-CD28 (*$p < 0.01$). (B) Whole-cell lysates were separated by SDS-PAGE (each 10 μg), and EGR2 was detected by immunoblotting. The same blots were stripped and reprobed with Abs specific for β-actin as a loading control. Data shown are representative of two independent experiments with similar results. (**C**) Freshly purified CD4$^+$ T cells were stimulated with anti-CD3 plus anti-CD28 mAbs (25 μg/ml), anti-CD3 plus anti-CD26 mAbs (25 μg/ml), or anti-CD3 mAb plus Cav-Ig (25 μg/ml) for 6 h in the presence of vehicle (DMSO, 0.025%), NFAT inhibitor (CyA, 1 μM), or NFAT inhibitor plus MEK1/2 inhibitor (U0126, 5 μM). mRNA expression of EGR2 was quantified by real-time RT-PCR, and the relative expression level is shown as in (A). Representative data of three independent donors are shown as mean ± SD of triplicate samples. (**D**) Freshly purified CD4$^+$ T cells were transfected with siRNA against EGR2 or control siRNA (Ctrl) and stimulated with anti-CD3 plus anti-CD28 mAbs (25 μg/ml) or anti-CD3 plus anti-CD26 mAbs (25 μg/ml) for 24 h (**a**) or 96 h (**b–d**). (Da) mRNA level of EGR2 is shown as in (A). (Db–d) Concentrations of IL-10 (b), IL-17A (c), and IFN-γ (d) were examined by ELISA. Representative data of four independent donors are shown as mean ± SD of triplicate samples, comparing values with EGR2 siRNA to those with control siRNA (*$p < 0.01$), and similar results were obtained in each experiment.
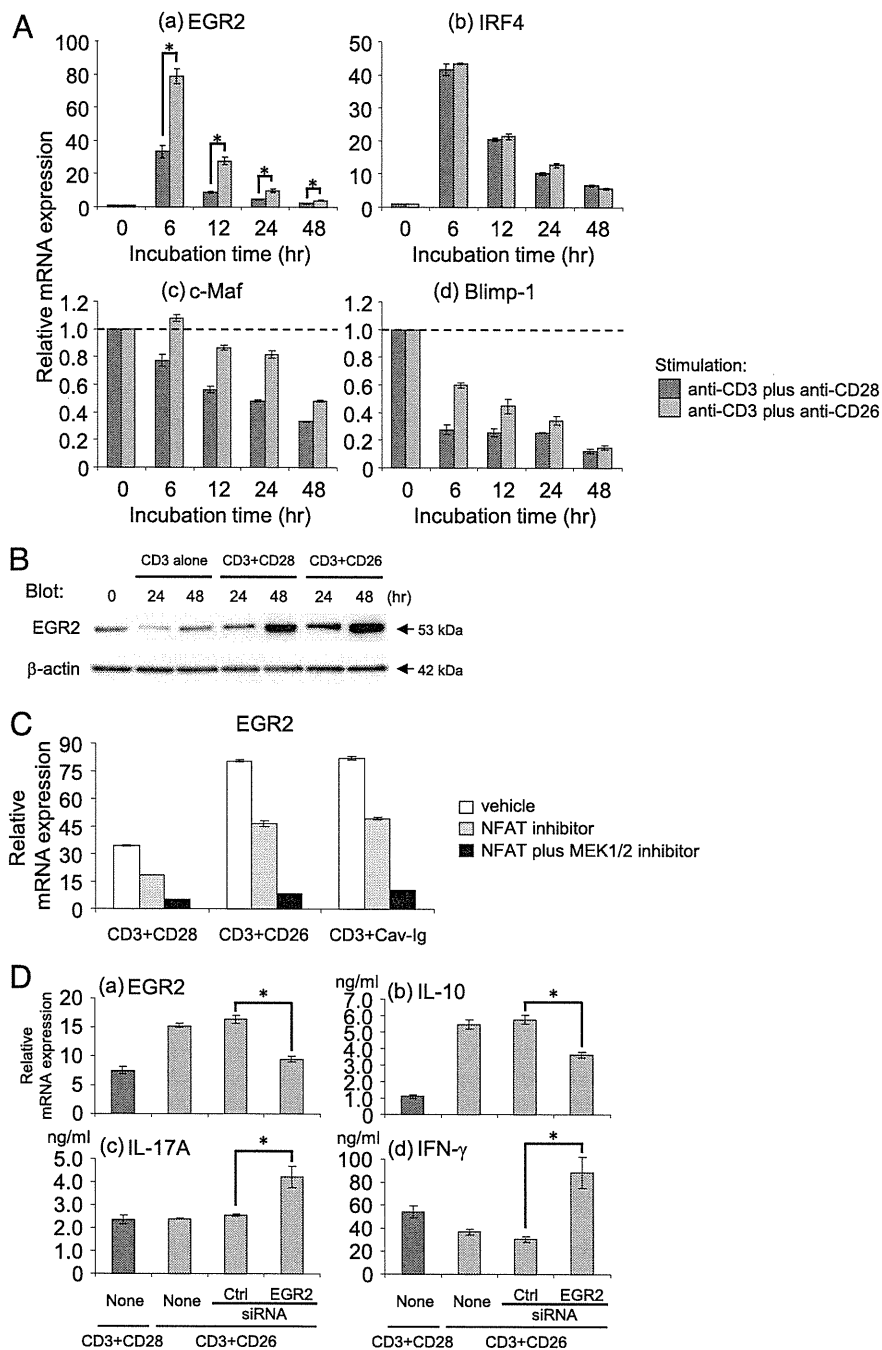
Discussion

In the present study, we show that coengagement of CD3 and CD26 induces the development of CD4$^+$ T cells to a Tr1-like phenotype with a high level of IL-10 production and LAG3 expression. CD26 costimulation also induces a high level of EGR2 associated with preferential IL-10 production, possibly via NFAT- and AP-1–mediated signaling. Furthermore, supernatants of these CD3/CD26-stimulated CD4$^+$ T cells clearly suppress the proliferative activity and effector cytokine production of bystander T cells in an IL-10–dependent manner.

Because the maintenance of peripheral tolerance by Tregs is critical to the potential development of autoimmunity, there is considerable interest in elucidating the molecular mechanisms for inducing the differentiation of CD4$^+$ T cells into specific Treg subsets. Although the mechanisms of Treg induction through exogenous cytokine stimulation have been extensively studied, such

as TGF-β for Foxp3$^+$ Tregs or IL-27 for Tr1 cells (41), costimulatory signals for the induction of Tregs have not been fully understood. ICOS and CD46 have been reported as costimulatory signals inducing Tr1-type cells (42, 43). ICOS costimulation regulates c-Maf expression possibly through the enhancement of NFAT2 signal (44). CD46 costimulation induces interaction of the cytoplasmic tail of CD46 (CYT-1-BC1) with SPAK, leading to sustained phosphorylation of ERK1/2 (45). The importance of sustained phosphorylation of ERK1/2 has also been reported for the development of IL-10–producing Th1 cells (46). However, sustained phosphorylation of ERK1/2 alone is not sufficient to explain the preferential IL-10 production, and transcription factors such as c-Maf, IRF4, Blimp-1, GATA-3, and E4BP4 (NFIL3) may be associated with the transcription of IL-10 (34–38). Our present study indicates that EGR2 expression is enhanced in CD4$^+$ T cells following CD26-mediated costimulation (Fig. 5A, B), and EGR2
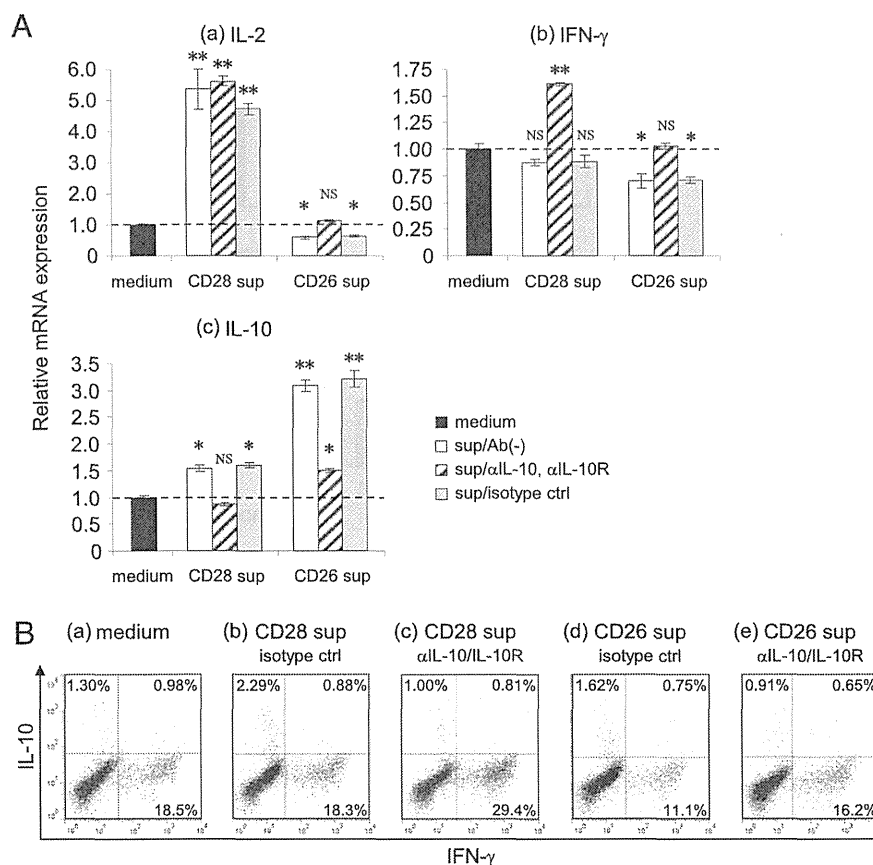
FIGURE 6. Supernatants of CD3/CD26-activated CD4$^+$ T cells suppress effector function of bystander T cells while augmenting IL-10 expression in an IL-10–dependent manner. Culture supernatants of CD4$^+$ T cells stimulated with anti-CD3 plus anti-CD28 mAbs (50 μg/ml) or anti-CD3 plus anti-CD26 mAbs (50 μg/ml) for 72 h were collected, and freshly purified CD4$^+$ T cells were stimulated with anti-CD3 plus anti-CD26 mAbs (5 μg/ml) in the presence of the supernatant (CD28 sup or CD26 sup) or AIM-V medium as a control. Prior to the onset of culture, the combination of anti-human IL-10 mAb and anti-human IL-10 receptor mAb (αIL10/IL10R) or isotype control mAbs (isotype ctrl) were added to the culture wells to give a final concentration of 20 μg/ml each. (**A**) After 24 h of incubation, cells were harvested and mRNA expression of IL-2 (**a**), IFN-γ (**b**), or IL-10 (**c**) was quantified by real-time RT-PCR. Each expression was normalized to HPRT1, and relative expression levels compared with the sample of control medium are shown. Representative data of three independent donors are shown as mean ± SD of triplicate samples, comparing values in each sample to that in control medium. $*p < 0.01$, $**p < 0.0001$. (**B**) On day 3, cells were restimulated with PMA plus ionomycin in the presence of monensin for the last 5 h of culture, and the intracellular expression of IFN-γ and IL-10 was detected by flow cytometry. Two-dimensional dot plot of IFN-γ or IL-10 staining gated for CD4$^+$ T cells is shown as a representative plot of three independent donors, and similar results were obtained in each experiment.

knockdown in CD4$^+$ T cells decreases IL-10 while markedly enhancing IL-17A and IFN-γ production (Fig. 5D). These observations strongly suggest that the induction of EGR2 expression is associated with the preferential production of IL-10 after CD26 costimulation. In mice, it has been recently reported that a high level of EGR2 expression is induced by IL-27/IL-27R–STAT3 signaling, and Blimp-1 induced by EGR2 is important for IL-10 production in CD4$^+$ T cells (47). In contrast with the marked upregulation of EGR2 following CD26-mediated costimulation, the expression level of Blimp-1 is not enhanced as compared with unstimulated T cells, although the decrease is more apparent in T cells following CD28 costimulation (Fig. 5A). To characterize more precisely the molecular mechanisms involved in IL-10 induction following CD26-mediated costimulation, the role of Blimp-1 or c-Maf needs to be better defined. NFAT is considered to be a regulator of EGR2 expression in T cells (39). Our present work shows that EGR2 is highly induced via CD26 costimulatory signal, and its expression is partially decreased by the NFAT inhibitor CyA and completely abrogated by the combination of CyA and the MEK1/2 inhibitor U0126 (Fig. 5C). These results strongly suggest that not only NFAT but also Raf-MEK-ERK signaling is involved in the induction of EGR2 expression. The association of

ERK1/2 signaling in the induction of EGR2 expression has been recently reported in osteoprogenitors or breast adipose fibroblasts (48, 49), suggesting that the ERK signaling pathway is also associated with the transcription of EGR2 in T cells.

The importance of the anti-inflammatory effects of IL-10 has been confirmed in IL-10–deficient mice. IL-10–deficient mice develop spontaneous colitis at an early age, indicating that IL-10 is essential for intestinal homeostasis (50). IL-10–deficient mice also exhibit severe neuroinflammation with loss of recovery in experimental autoimmune encephalomyelitis (51). Furthermore, the generation of Tr1 cells from peripheral CD4$^+$ T cells has been shown to be greatly impaired in MS patients in comparison with healthy controls (52). Therefore, Tr1-mediated immunotherapy may be a potentially effective approach for the treatment of many autoimmune disorders, including inflammatory bowel disease or MS. Several groups have attempted to generate large numbers of Tr1 in vitro for clinical applications, and IL-27 is being considered currently to be an essential factor for the generation of Tr1 cells (53). In addition to IL-27, costimulatory signals through ICOS or CD46 play an important role in the generation or expansion of Tr1 cells. Our present study raises the possibility that CD26 is a novel costimulatory molecule inducing preferential IL-10 production in
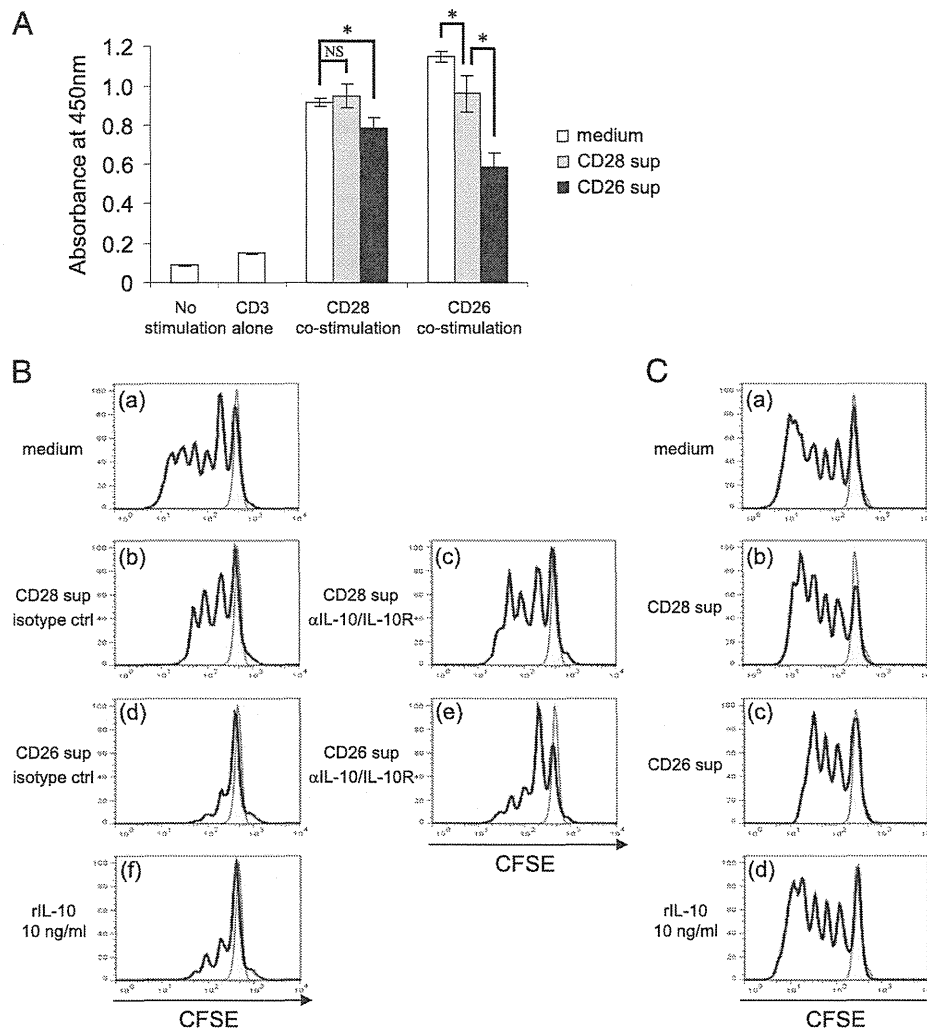
FIGURE 7. Supernatants of CD3/CD26-activated CD4$^+$ T cells significantly suppress the proliferation of bystander T cells through partial dependence on IL-10. Culture supernatants were prepared by the same method as shown in Fig. 6. (**A**) Freshly purified CD4$^+$ T cells were stimulated with anti-CD3 mAb alone, anti-CD3 plus anti-CD28 mAbs (5 μg/ml), or anti-CD3 plus anti-CD26 mAbs (5 μg/ml) for 96 h in the presence of the supernatant (CD28 sup or CD26 sup) or AIM-V medium as a control. Tetrazolium was added for the last 3 h of culture, and the absorbance at 450 nm was measured. Representative data of five independent donors are shown as mean ± SD of triplicate samples, comparing values with culture supernatants to those with control medium (*$p < 0.01$). (**B**) CFSE-labeled freshly purified CD4$^+$ T cells were stimulated with anti-CD3 plus anti-CD26 mAbs (5 μg/ml) in the presence of the supernatant [CD28 sup (**b** and **c**) or CD26 sup (**d** and **e**)] or recombinant human IL-10 (rIL-10, 10 ng/ml) (**f**) or AIM-V medium (**a**) as a control. Prior to the onset of culture, the combination of anti-human IL-10 mAb and anti-human IL-10 receptor mAb (αIL-10/IL-10R) (Bc and e) or isotype control mAbs (isotype ctrl) (Bb and d) were added to the culture wells to give a final concentration of 20 μg/ml each. (**C**) CFSE-labeled freshly purified CD4$^+$ T cells were stimulated with anti-CD3 plus anti-CD28 mAbs (5 μg/ml) in the presence of the supernatant [CD28 sup (**b**) or CD26 sup (**c**)] or recombinant human IL-10 (rIL-10, 10 ng/ml) (**d**) or AIM-V medium (**a**) as a control. After 96 h of incubation, cells were harvested and analyzed by flow cytometry. The data are shown as histograms of CFSE intensity gated for CD4$^+$ T cells and are representative of three independent donors, and similar results were obtained in each experiment. The gray areas in each histogram show the data of T cells cultured for 96 h without any stimulation.

CD4$^+$ T cells. Because CD26, ICOS, and CD46 belong to different families and their downstream signaling events are considered to be different, the combination of these costimulatory pathways may have additive effects on the induction of Tr1 cells. For this purpose, identification of the T cell subsets producing a high level of IL-10 following CD26 costimulation is needed. Because the CD4$^+$ T cells used in this study contained all the T cell subsets such as naive, Th1, Th2, Th17, follicular helper T, or Treg, we conducted costimulation assays following purification of CD4$^+$ naive, memory, or Treg subsets. Although the absolute amount of IL-10 produced by CD45RO$^+$ memory T cells was higher than CD45RA$^+$ naive T cells, both naive and memory CD4$^+$ T cells costimulated through CD26 produced greater levels of IL-10 compared with CD28 costimulation (Supplemental Fig. 3). In contrast, purified CD4$^+$CD25^{high}CD127^{low}FOXP3$^+$ cells appar-

ently produced a low level of IL-10 following CD26 costimulation compared with other populations of CD4$^+$ T cells (Supplemental Fig. 3). These observations indicate that naturally occurring CD4$^+$ CD25^{high}CD127^{low}FOXP3$^+$ cells are not the source of IL-10 production, and they suggest that the subpopulation included in memory CD4$^+$ T cells is the main source of IL-10, whereas naive CD4$^+$ T cells also produce IL-10 in response to CD26 costimulation. Additionally, in vivo studies focusing on the suppressive activity of this population of in vitro–differentiated CD4$^+$ T cells, or the stability or plasticity of this IL-10–producing phenotype, need to be considered in future work.

CD28 is a representative T cell costimulatory pathway, and the negative feedback mechanism through CTLA-4 is associated with this pathway as a means of controlling excessive T cell activation. CD26 also functions as a costimulatory molecule in human T cells,

and CD26$^+$ T cells have been suggested to be involved in the pathophysiology of various immune disorders such as MS, rheumatoid arthritis, and graft-versus-host disease (8, 10, 12). Our present study shows that stimulation through TCR and high-intensity interaction of caveolin-1–CD26-mediated signaling induces the development of CD4$^+$ T cells to a Tr1-like phenotype. Caveolin-1 is a ubiquitously expressed ligand of CD26 in many cell types such as epithelial cells, endothelial cells, fibroblasts, macrophages, and neutrophils. We have previously shown that caveolin-1 was detected on the cell surface of monocytes 12–24 h after Ag uptake, and that CD26 and caveolin-1 colocalized at the T cell/monocyte contact site (19). Moreover, caveolin-1 expression is regulated by NF-κB, and stimulation with LPS or TNF-α increases the expression of caveolin-1 mRNA and protein (54), suggesting that expression of caveolin-1 may be increased on cells accumulated at sites of inflammation, leading to the transduction of intensive CD26-mediated signaling in CD26$^+$ T cells. Taken together, our data strongly suggest that CD4$^+$ T cells receiving robust caveolin-1–CD26-mediated signaling at the inflammatory site produce a high level of IL-10 and potentially other inhibitory factors to curtail the inflammatory process. Because striking defects in the induction of Tr1 cells through CD46 costimulation have been reported in patients with MS (52), it is conceivable that negative feedback mechanisms for regulating excessive CD26-mediated activation may be impaired in patients with autoimmune diseases, and further research is required to evaluate this hypothesis.

The cytoplasmic domain of CD28 has several common motifs that bind to signaling molecules such as PI3K, growth factor receptor–bound protein 2, or IL-2–inducible T cell kinase, whereas the cytoplasmic tail of CD26 consists of only 6 aa without any conserved kinase or protein-binding motifs (18). We have previously shown that CD26 localizes into lipid rafts, and stimulation with anti-CD3 plus anti-CD26 promotes aggregation of lipid rafts, leading to colocalization of CD45 to TCR signaling molecules such as p56^{Lck}, ZAP-70, or TCRζ (30). Our present data strongly suggest that persistent NFAT–AP-1 cooperation is responsible for CD26-mediated T cell activation, and sustained activation of NFAT and ERK1/2 is possibly due to the aggregation of signaling molecules in lipid rafts following CD26-mediated costimulation. Furthermore, we have previously shown that DPPIV enzyme activity is partially involved in the costimulatory activity of CD26 through studies using wild-type CD26 (DPPIV$^+$) or mutant CD26 (DPPIV$^-$)–transfected Jurkat T cell lines (55). Other groups reported that the synthetic competitive DPPIV inhibitor Lys[Z(NO$_2$)] significantly suppressed the proliferation and production of IL-2, IL-10, and IFN-γ in PWM-stimulated human T cells (56). They showed that these DPPIV inhibitors markedly increased the secretion of latent TGF-β1 by PWM-stimulated T cells, resulting in suppression of T cell activation. To further characterize the role of DPPIV enzyme activity in T cell activation, we are now examining the effect of the clinically used DPPIV specific inhibitor sitagliptin. Our preliminary data indicate that sitagliptin strongly suppresses the proliferative activity and production of cytokines including IL-10 of human CD4$^+$ T cells following CD26-mediated costimulation compared with CD28-mediated costimulation (data not shown), with the precise molecular mechanisms involved in this process being currently investigated.

In conclusion, intensive CD26 costimulatory signaling induces the development of CD4$^+$ T cells to a Tr1-like phenotype with a high level of IL-10 production, possibly for regulating potentially excessive CD26-mediated activation. Whether this possible negative feedback mechanism of CD26 costimulation is normally maintained or impaired in patients with autoimmune diseases is the subject of future investigations.

Disclosures

References

1. Fox, D. A., R. E. Hussey, K. A. Fitzgerald, O. Acuto, C. Poole, L. Palley, J. F. Daley, S. F. Schlossman, and E. L. Reinherz. 1984. Ta1, a novel 105 KD human T cell activation antigen defined by a monoclonal antibody. *J. Immunol.* 133: 1250–1256.
2. Nanus, D. M., D. Engelstein, G. A. Gastl, L. Gluck, M. J. Vidal, M. Morrison, C. L. Finstad, N. H. Bander, and A. P. Albino. 1993. Molecular cloning of the human kidney differentiation antigen gp160: human aminopeptidase A. *Proc. Natl. Acad. Sci. USA* 90: 7069–7073.
3. Tanaka, T., D. Camerini, B. Seed, Y. Torimoto, N. H. Dang, J. Kameoka, H. N. Dahlberg, S. F. Schlossman, and C. Morimoto. 1992. Cloning and functional expression of the T cell activation antigen CD26. *J. Immunol.* 149: 481–486.
4. Morimoto, C., and S. F. Schlossman. 1998. The structure and function of CD26 in the T-cell immune response. *Immunol. Rev.* 161: 55–70.
5. Ohnuma, K., N. H. Dang, and C. Morimoto. 2008. Revisiting an old acquaintance: CD26 and its molecular mechanisms in T cell function. *Trends Immunol.* 29: 295–301.
6. Morimoto, C., Y. Torimoto, G. Levinson, C. E. Rudd, M. Schrieber, N. H. Dang, N. L. Letvin, and S. F. Schlossman. 1989. 1F7, a novel cell surface molecule, involved in helper function of CD4 cells. *J. Immunol.* 143: 3430–3439.
7. Bengsch, B., B. Seigel, T. Flecken, J. Wolanski, H. E. Blum, and R. Thimme. 2012. Human Th17 cells express high levels of enzymatically active dipeptidylpeptidase IV (CD26). *J. Immunol.* 188: 5438–5447.
8. Krakauer, M., P. S. Sorensen, and F. Sellebjerg. 2006. CD4$^+$ memory T cells with high CD26 surface expression are enriched for Th1 markers and correlate with clinical severity of multiple sclerosis. *J. Neuroimmunol.* 181: 157–164.
9. Eguchi, K., Y. Ueki, C. Shimomura, T. Otsubo, H. Nakao, K. Migita, A. Kawakami, M. Matsunaga, H. Tezuka, N. Ishikawa, et al. 1989. Increment in the Ta1$^+$ cells in the peripheral blood and thyroid tissue of patients with Graves' disease. *J. Immunol.* 142: 4233–4240.
10. Ohnuma, K., H. Inoue, M. Uchiyama, T. Yamochi, O. Hosono, N. H. Dang, and C. Morimoto. 2006. T-cell activation via CD26 and caveolin-1 in rheumatoid synovium. *Mod. Rheumatol.* 16: 3–13.
11. Hatano, R., K. Ohnuma, J. Yamamoto, N. H. Dang, and C. Morimoto. 2013. CD26-mediated co-stimulation in human CD8$^+$ T cells provokes effector function via pro-inflammatory cytokine production. *Immunology* 138: 165–172.
12. Hatano, R., K. Ohnuma, J. Yamamoto, N. H. Dang, T. Yamada, and C. Morimoto. 2013. Prevention of acute graft-versus-host disease by humanized anti-CD26 monoclonal antibody. *Br. J. Haematol.* 162: 263–277.
13. Bluestone, J. A. 1995. New perspectives of CD28-B7-mediated T cell costimulation. *Immunity* 2: 555–559.
14. Shahinian, A., K. Pfeffer, K. P. Lee, T. M. Kündig, K. Kishihara, A. Wakeham, K. Kawai, P. S. Ohashi, C. B. Thompson, and T. W. Mak. 1993. Differential T cell costimulatory requirements in CD28-deficient mice. *Science* 261: 609–612.
15. Tivol, E. A., F. Borriello, A. N. Schweitzer, W. P. Lynch, J. A. Bluestone, and A. H. Sharpe. 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* 3: 541–547.
16. Waterhouse, P., J. M. Penninger, E. Timms, A. Wakeham, A. Shahinian, K. P. Lee, C. B. Thompson, H. Griesser, and T. W. Mak. 1995. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science* 270: 985–988.
17. Stamper, C. C., Y. Zhang, J. F. Tobin, D. V. Erbe, S. Ikemizu, S. J. Davis, M. L. Stahl, J. Seehra, W. S. Somers, and L. Mosyak. 2001. Crystal structure of the B7-1/CTLA-4 complex that inhibits human immune responses. *Nature* 410: 608–611.
18. Rudd, C. E., and H. Schneider. 2003. Unifying concepts in CD28, ICOS and CTLA4 co-receptor signalling. *Nat. Rev. Immunol.* 3: 544–556.
19. Ohnuma, K., T. Yamochi, M. Uchiyama, K. Nishibashi, N. Yoshikawa, N. Shimizu, S. Iwata, H. Tanaka, N. H. Dang, and C. Morimoto. 2004. CD26 upregulates expression of CD86 on antigen-presenting cells by means of caveolin-1. *Proc. Natl. Acad. Sci. USA* 101: 14186–14191.
20. Ohnuma, K., M. Uchiyama, T. Yamochi, K. Nishibashi, O. Hosono, N. Takahashi, S. Kina, H. Tanaka, X. Lin, N. H. Dang, and C. Morimoto. 2007. Caveolin-1 triggers T-cell activation via CD26 in association with CARMA1. *J. Biol. Chem.* 282: 10117–10131.
21. Stremenová, J., V. Mares, V. Lisá, M. Hilser, E. Krepela, Z. Vanicková, M. Syrucek, O. Soula, and A. Sedo. 2010. Expression of dipeptidyl peptidase-IV activity and/or structure homologs in human meningiomas. *Int. J. Oncol.* 36: 351–358.
22. Nakamura, K., A. Kitani, and W. Strober. 2001. Cell contact-dependent immunosuppression by CD4$^+$CD25$^+$ regulatory T cells is mediated by cell surface-bound transforming growth factor β. *J. Exp. Med.* 194: 629–644.
23. Collison, L. W., C. J. Workman, T. T. Kuo, K. Boyd, Y. Wang, K. M. Vignali, R. Cross, D. Sehy, R. S. Blumberg, and D. A. Vignali. 2007. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* 450: 566–569.
24. Groux, H., A. O'Garra, M. Bigler, M. Rouleau, S. Antonenko, J. E. de Vries, and M. G. Roncarolo. 1997. A CD4$^+$ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature* 389: 737–742.
25. Okamura, T., K. Fujio, M. Shibuya, S. Sumitomo, H. Shoda, S. Sakaguchi, and K. Yamamoto. 2009. CD4$^+$CD25$^-$LAG3$^+$ regulatory T cells controlled by the transcription factor Egr-2. *Proc. Natl. Acad. Sci. USA* 106: 13974–13979.

26. Huard, B., P. Prigent, F. Pagès, D. Bruniquel, and F. Triebel. 1996. T cell major histocompatibility complex class II molecules down-regulate CD4+ T cell clone responses following LAG-3 binding. *Eur. J. Immunol.* 26: 1180–1186.

27. Maçon-Lemaître, L., and F. Triebel. 2005. The negative regulatory function of the lymphocyte-activation gene-3 co-receptor (CD223) on human T cells. *Immunology* 115: 170–178.

28. Zheng, Y., Y. Zha, G. Driessens, F. Locke, and T. F. Gajewski. 2012. Transcriptional regulator early growth response gene 2 (Egr2) is required for T cell anergy in vitro and in vivo. *J. Exp. Med.* 209: 2157–2163.

29. Hatano, R., T. Yamada, S. Matsuoka, S. Iwata, H. Yamazaki, E. Komiya, T. Okamoto, N. H. Dang, K. Ohnuma, and C. Morimoto. 2014. Establishment of monoclonal anti-human CD26 antibodies suitable for immunostaining of formalin-fixed tissue. *Diagn. Pathol.* 9: 30.

30. Ishii, T., K. Ohnuma, A. Murakami, N. Takasawa, S. Kobayashi, N. H. Dang, S. F. Schlossman, and C. Morimoto. 2001. CD26-mediated signaling for T cell activation occurs in lipid rafts through its association with CD45RO. *Proc. Natl. Acad. Sci. USA* 98: 12138–12143.

31. Jonuleit, H., E. Schmitt, M. Stassen, A. Tuettenberg, J. Knop, and A. H. Enk. 2001. Identification and functional characterization of human CD4+CD25+ T cells with regulatory properties isolated from peripheral blood. *J. Exp. Med.* 193: 1285–1294.

32. Okamura, T., K. Fujio, S. Sumitomo, and K. Yamamoto. 2012. Roles of LAG3 and EGR2 in regulatory T cells. *Ann. Rheum. Dis.* 71(Suppl. 2): i96–i100.

33. Macian, F. 2005. NFAT proteins: key regulators of T-cell development and function. *Nat. Rev. Immunol.* 5: 472–484.

34. Xu, J., Y. Yang, G. Qiu, G. Lal, Z. Wu, D. E. Levy, J. C. Ochando, J. S. Bromberg, and Y. Ding. 2009. c-Maf regulates IL-10 expression during Th17 polarization. *J. Immunol.* 182: 6226–6236.

35. Ahyi, A. N., H. C. Chang, A. L. Dent, S. L. Nutt, and M. H. Kaplan. 2009. IFN regulatory factor 4 regulates the expression of a subset of Th2 cytokines. *J. Immunol.* 183: 1598–1606.

36. Cretney, E., A. Xin, W. Shi, M. Minnich, F. Masson, M. Miasari, G. T. Belz, G. K. Smyth, M. Busslinger, S. L. Nutt, and A. Kallies. 2011. The transcription factors Blimp-1 and IRF4 jointly control the differentiation and function of effector regulatory T cells. *Nat. Immunol.* 12: 304–311.

37. Shoemaker, J., M. Saraiva, and A. O'Garra. 2006. GATA-3 directly remodels the IL-10 locus independently of IL-4 in CD4+ T cells. *J. Immunol.* 176: 3470–3479.

38. Motomura, Y., H. Kitamura, A. Hijikata, Y. Matsunaga, K. Matsumoto, H. Inoue, K. Atarashi, S. Hori, H. Watarai, J. Zhu, et al. 2011. The transcription factor E4BP4 regulates the production of IL-10 and IL-13 in CD4+ T cells. *Nat. Immunol.* 12: 450–459.

39. Safford, M., S. Collins, M. A. Lutz, A. Allen, C. T. Huang, J. Kowalski, A. Blackford, M. R. Horton, C. Drake, R. H. Schwartz, and J. D. Powell. 2005. Egr-2 and Egr-3 are negative regulators of T cell activation. *Nat. Immunol.* 6: 472–480.

40. Zhu, B., A. L. Symonds, J. E. Martin, D. Kioussis, D. C. Wraith, S. Li, and P. Wang. 2008. Early growth response gene 2 (Egr-2) controls the self-tolerance of T cells and prevents the development of lupuslike autoimmune disease. *J. Exp. Med.* 205: 2295–2307.

41. Sabat, R., G. Grütz, K. Warszawska, S. Kirsch, E. Witte, K. Wolk, and J. Geginat. 2010. Biology of interleukin-10. *Cytokine Growth Factor Rev.* 21: 331–344.

42. Hutloff, A., A. M. Dittrich, K. C. Beier, B. Eljaschewitsch, R. Kraft, I. Anagnostopoulos, and R. A. Kroczek. 1999. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. *Nature* 397: 263–266.

43. Kemper, C., A. C. Chan, J. M. Green, K. A. Brett, K. M. Murphy, and J. P. Atkinson. 2003. Activation of human CD4+ cells with CD3 and CD46 induces a T-regulatory cell 1 phenotype. *Nature* 421: 388–392.

44. Nurieva, R. I., J. Duong, H. Kishikawa, U. Dianzani, J. M. Rojo, Ic. Ho, R. A. Flavell, and C. Dong. 2003. Transcriptional regulation of th2 differentiation by inducible costimulator. *Immunity* 18: 801–811.

45. Cardone, J., G. Le Friec, P. Vantourout, A. Roberts, A. Fuchs, I. Jackson, T. Suddason, G. Lord, J. P. Atkinson, A. Cope, et al. 2010. Complement regulator CD46 temporally regulates cytokine production by conventional and unconventional T cells. *Nat. Immunol.* 11: 862–871.

46. Saraiva, M., J. R. Christensen, M. Veldhoen, T. L. Murphy, K. M. Murphy, and A. O'Garra. 2009. Interleukin-10 production by Th1 cells requires interleukin-12-induced STAT4 transcription factor and ERK MAP kinase activation by high antigen dose. *Immunity* 31: 209–219.

47. Iwasaki, Y., K. Fujio, T. Okamura, A. Yanai, S. Sumitomo, H. Shoda, T. Tamura, H. Yoshida, P. Charnay, and K. Yamamoto. 2013. Egr-2 transcription factor is required for Blimp-1-mediated IL-10 production in IL-27-stimulated CD4+ T cells. *Eur. J. Immunol.* 43: 1063–1073.

48. Chandra, A., S. Lan, J. Zhu, V. A. Siclari, and L. Qin. 2013. Epidermal growth factor receptor (EGFR) signaling promotes proliferation and survival in osteoprogenitors by increasing early growth response 2 (EGR2) expression. *J. Biol. Chem.* 288: 20488–20498.

49. To, S. Q., K. C. Knower, and C. D. Clyne. 2013. NFκB and MAPK signalling pathways mediate TNFα-induced early growth response gene transcription leading to aromatase expression. *Biochem. Biophys. Res. Commun.* 433: 96–101.

50. Kühn, R., J. Löhler, D. Rennick, K. Rajewsky, and W. Müller. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 75: 263–274.

51. Bettelli, E., M. P. Das, E. D. Howard, H. L. Weiner, R. A. Sobel, and V. K. Kuchroo. 1998. IL-10 is critical in the regulation of autoimmune encephalomyelitis as demonstrated by studies of IL-10- and IL-4-deficient and transgenic mice. *J. Immunol.* 161: 3299–3306.

52. Astier, A. L., G. Meiffren, S. Freeman, and D. A. Hafler. 2006. Alterations in CD46-mediated Tr1 regulatory T cells in patients with multiple sclerosis. *J. Clin. Invest.* 116: 3252–3257.

53. Vasanthakumar, A., and A. Kallies. 2013. IL-27 paves different roads to Tr1. *Eur. J. Immunol.* 43: 882–885.

54. Tiruppathi, C., J. Shimizu, K. Miyawaki-Shimizu, S. M. Vogel, A. M. Bair, R. D. Minshall, D. Predescu, and A. B. Malik. 2008. Role of NF-κB-dependent caveolin-1 expression in the mechanism of increased endothelial permeability induced by lipopolysaccharide. *J. Biol. Chem.* 283: 4210–4218.

55. Tanaka, T., J. Kameoka, A. Yaron, S. F. Schlossman, and C. Morimoto. 1993. The costimulatory activity of the CD26 antigen requires dipeptidyl peptidase IV enzymatic activity. *Proc. Natl. Acad. Sci. USA* 90: 4586–4590.

56. Reinhold, D., U. Bank, F. Bühling, U. Lendeckel, J. Faust, K. Neubert, and S. Ansorge. 1997. Inhibitors of dipeptidyl peptidase IV induce secretion of transforming growth factor-β1 in PWM-stimulated PBMC and T cells. *Immunology* 91: 354–360.

RESEARCH ARTICLE

Clinical Significance of Soluble CD26 in Malignant Pleural Mesothelioma

Nobukazu Fujimoto[1]*, Kei Ohnuma[2], Keisuke Aoe[3], Osamu Hosono[2], Taketo Yamada[4], Takumi Kishimoto[5], Chikao Morimoto[2]

1. Department of Medical Oncology, Okayama Rosai Hospital, Okayama, Japan, 2. Department of Therapy Development and Innovation for Immune Disorders and Cancers, Graduate School of Medicine, Juntendo University, Hongo, Bunkyo-ku, Tokyo, Japan, 3. Department of Medical Oncology and Clinical Research, National Hospital Organization Yamaguchi-Ube Medical Center, Ube, Yamaguchi, Japan, 4. Department of Pathology, Keio University school of Medicine, Tokyo, Japan, 5. Department of Internal Medicine, Okayama Rosai Hospital, Okayama, Japan

*nobufujimot@gmail.com

Abstract

There is no established single diagnostic marker for malignant pleural mesothelioma (MPM). CD26 is a 110 kDa, multifunctional, membrane-bound glycoprotein that has dipeptidyl peptidase IV (DPPIV) enzyme activity. The aim of this study was to evaluate the clinical significance of soluble CD26 (sCD26) in patients with MPM. The study included 80 MPM patients, 79 subjects with past asbestos exposure (SPE), and 134 patients with other benign pleural diseases (OPD) that were included as a control group. sCD26 levels and DPPIV activity in serum and/or pleural fluid were determined using an ELISA kit. Serum sCD26 levels and DPPIV enzyme activity in patients with MPM were significantly decreased compared with those in the SPE group (P=0.000). The level of serum sCD26 was significantly decreased in patients with advanced stages of MPM compared with those with earlier stages (P=0.047). The median OS of patients with MPM who had higher DPPIV enzyme activity was significantly longer than that of those with lower DPPIV enzyme activity (P=0.032). The sCD26 levels in the pleural fluid of MPM patients with an epithelioid subtype were significantly increased compared with the OPD cohort (P=0.012). Moreover, DPPIV enzyme activity in the pleural fluid of patients with MPM with an epithelioid subtype were significantly increased compared with those in the OPD cohort (P=0.009). Patients with MPM who had lower specific DPPIV activity, determined as DPPIV/sCD26, showed significantly prolonged survival compared with those with higher specific DPPIV activity (P=0.028). Serum sCD26 and DPPIV enzyme activity appear to be useful biomarkers for differentiating patients with MPM from SPE. The sCD26 levels or DPPIV enzyme activity in pleural fluid appear to be biomarkers in patients with an

epithelioid subtype of MPM. DPPIV activity in serum or pleural fluid appears to be predictive for the prognosis of patients with MPM.

Introduction

Malignant pleural mesothelioma (MPM) is an aggressive malignancy arising from the mesothelial cells lining the pleura [1]. It is generally associated with a history of asbestos exposure [2] and has a very poor prognosis [3]. Once rare, the incidence of MPM has increased in industrialized nations including Japan and the United States as a result of past wide-spread exposure to asbestos [4]. The incidence of MPM is predicted to increase in the next decades, especially in developing countries where asbestos has not yet been banned [1,4,5]. Treatment for MPM includes surgery, radiotherapy, and/or systemic chemotherapy, but the effectiveness of these interventions is limited. Therefore, novel strategies for early diagnosis and screening of people with past asbestos exposure who are at high risk are urgently needed to improve the outcome.

There is presently no established single diagnostic marker of clinical significance for MPM. Soluble mesothelin-related peptides (SMRP) appear promising for differentiating MPM from lung cancer (LC) [6,7]. Recently, Shiomi et al reported that N-ERC/mesothelin may be a useful marker for diagnosing MPM [8]. Pass et al reported that plasma fibulin-3 levels could distinguish healthy persons with exposure to asbestos from patients with MPM [9]. However, these markers have not yet been established for use in clinical practice.

CD26 is a 110 kDa, multifunctional, membrane-bound glycoprotein, with dipeptidyl peptidase IV (DPPIV) enzyme activity in its extracellular domain [10] and is critical in T-cell biology as a marker of T-cell activation [11–13]. CD26 has an important but complex function in tumor behavior. Its biological effect depends on the tumor type and microenvironment. It is a marker of aggressive disease for certain subsets of T-cell non-Hodgkin's lymphomas/leukemias where expression of CD26 on T-lymphoblastic lymphomas/acute lymphoblastic leukemia cells is associated with a worse outcome compared with CD26-negative tumors [14]. CD26 is also expressed at high levels on renal carcinoma cells [15–17]. Recently, we showed that CD26 is preferentially expressed on malignant mesothelioma cells, but not on normal mesothelial cells. More importantly, humanized anti-CD26 antibody inhibited the growth of malignant mesothelioma cells and induced long-term survival of tumor-transplanted SCID mice [18]. More recently, we planned a treatment outcome prediction study and showed that CD26 membrane expression on MPM cells was closely correlated with responsiveness of the disease to chemotherapy [19]. All these findings suggest that CD26 would be a significant biomarker of MPM.

In the current study, we determined soluble CD26 (sCD26) and DPPIV enzyme activity in the serum and pleural fluid of patients with MPM. The aim of this

study was to evaluate the clinical significance of sCD26 as a screening, early diagnosis, and/or prognostic marker of MPM.

Materials and Methods

Subjects

The study included 80 MPM patients diagnosed and treated at Okayama Rosai Hospital and National Hospital Organization Yamaguchi-Ube Medical Center between 1998 and 2013. Histological sections from the patients with mesothelioma were examined and classified by immunohistochemistry as epithelioid, biphasic, or sarcomatous subtypes according to the World Health Organization histological classification [20]. Clinical stage was determined according to the criteria of the International Mesothelioma Interest Group TNM staging system for MPM [21]. Seventy-nine subjects with past asbestos exposure (SPE) and pleural plaques seen on chest computed tomography, and 134 patients with other benign pleural diseases (OPD) as a control group were also included. Portions of MPM and OPD were previously reported in our previous studies of SMRP [7] and hyaluronic acid determination [22]. Written informed consent was obtained from all patients.

Measurement of sCD26 or DPPIV Enzyme Activity

Serum samples were collected from 41 (29 epithelioid, 4 sarcomatous, and 8 biphasic) out of 80 patients with MPM, and from all those with SPE. Pleural fluid samples were collected from 65 (43 epithelioid, 15 biphasic, 7 sarcomatous) out of 80 patients with MPM, and all patients with OPD. The current study was initiated by determining sCD26 in pleural fluid. Subsequently, we added the analyses of serum sCD26. That is why there was lost data of MPM patients. For measurement of serum sCD26 levels or DPPIV enzyme activity, the serum or fluid samples were collected and stored at −80°C until measurement. Methods for measuring sCD26 and DPPIV enzyme activity were developed in our laboratory and have been described in detail elsewhere [23].

Measurement of SMRP

SMRP was measured by the chemiluminescent enzyme immunoassay (CLEIA) (Fujirebio Diagnostics. Malven, USA) based on 2-step sandwich method described in detail elsewhere [7].

Statistical Analysis

The results are shown as numbers (n), medians ± standard deviation (SD), or medians and interquartile range. Differences in means for laboratory data were analyzed by analysis of variance for multiple comparisons or two-tailed Student's t test for group comparisons. Areas under the receiver operating characteristic

(ROC) curves (AUCs) were calculated using standard techniques. Overall survival (OS) of patients with MPM was defined as the time from the day of diagnosis to the date of death or last follow-up. The proportion of survival and 95% confidence intervals (CI) were determined based on the Kaplan-Meyer method. Correlation was calculated as Pearson product-moment correlation coefficient. Statistical calculations were performed using the IBM SPSS Advanced Statistics19 (IBM Japan, Tokyo, Japan). All reported P values are two-sided. A level of P <0.05 was accepted as statistically significant.

Study Approval

Human study protocols were approved by the Ethics Committees at Okayama Rosai Hospital, National Hospital Organization Yamaguchi-Ube Medical Center, and Juntendo University. All studies on human subjects were carried out according to the principles set out in the Declaration of Helsinki.

Results

Demographic and Clinical Characteristics of the Study Populations

Of the 80 patients with MPM, the median (years $\pm$ SD) age was 69 ($\pm$9.13) years, and 75 were males and 5 were females. An occupational history of asbestos exposure was indicated in 75 patients and the median ($\pm$ SD) duration of asbestos exposure was 34 ($\pm$13.75) years. Of the group of 79 SPE, the median ($\pm$ SD) age was 66 ($\pm$5.50) years, 78 were males and 1 was female, and the median duration of asbestos exposure ($\pm$ SD) was 23 ($\pm$12.87) years. Of the 134 OPD patients, the median ($\pm$ SD) age was 76 ($\pm$10.34) years and 122 were males and 12 were females. The median age was significantly higher (P=0.000) and there were significantly more female patients (P=0.000) in OPD patients than in other groups.

Serum sCD26 and DPPIV Activity of Each Cohort

The median and interquartile range values of the serum and pleural fluid sCD26 levels and DPPIV enzyme activity are shown in S1 Table. To determine whether or not the serum levels of sCD26 or DPPIV enzyme activity were biomarkers among MPM patients, we first analyzed the differences in the serum levels of sCD26 or DPPIV enzyme activity between the MPM and SPE cohorts. As shown in Fig. 1A, serum sCD26 levels in patients with MPM were significantly decreased compared with the SPE group (P=0.000). To further clarify the usefulness of serum sCD26 levels for differentiating MPM from SPE, we performed a ROC analysis. The AUC value for the differential diagnosis between these 2 groups was 0.775 (95% CI, 0.682–0.868) (Fig. 1B). Based on a cutoff value of 1.00 μg/ml, the sensitivity was 74.7% and the specificity was 71.4% (Fig. 1B).

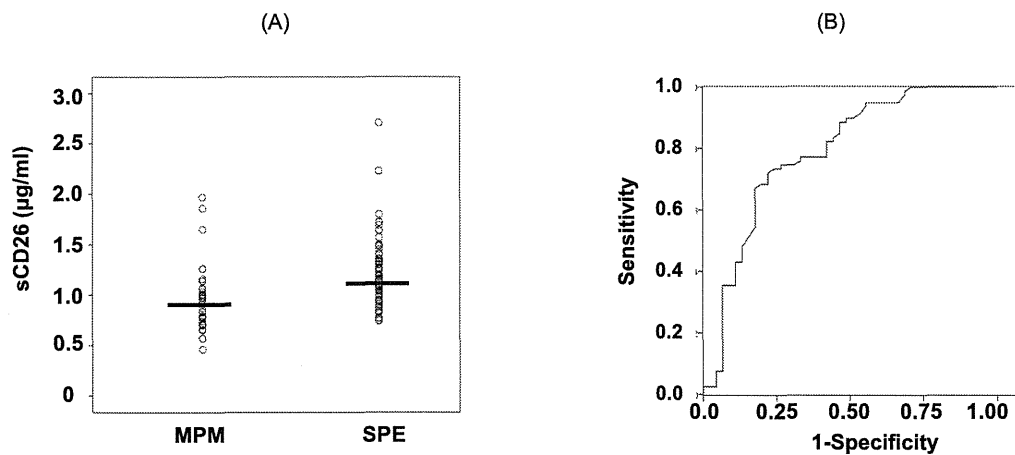(A)   (B)

Fig. 1. Comparison of serum soluble CD26 (sCD26) levels. (A) Comparison of serum sCD26 levels in patients with malignant pleural mesothelioma (MPM) or subjects with past asbestos exposure (SPE). Each dot indicates an individual value and the horizontal bar indicates the median value. (B) Receiver operating curve analysis of sCD26 levels according to the differentiation between patients with MPM and SPE.

sCD26 possesses DPPIV enzyme activity, which cleaves cytokines, chemokines, or peptide hormones to regulate their actions [10]. We examined the serum DPPIV enzyme activity to determine its usefulness as a biomarker for differentiating MPM from SPE. As shown in Fig. 2A, serum DPPIV enzyme activity is significantly decreased in patients with MPM compared with those with SPE (P=0.000). The ROC curve shows that the AUC value for the differential diagnosis of these 2 groups was 0.778 (95% CI, 0.690-0.865). Based on a cutoff
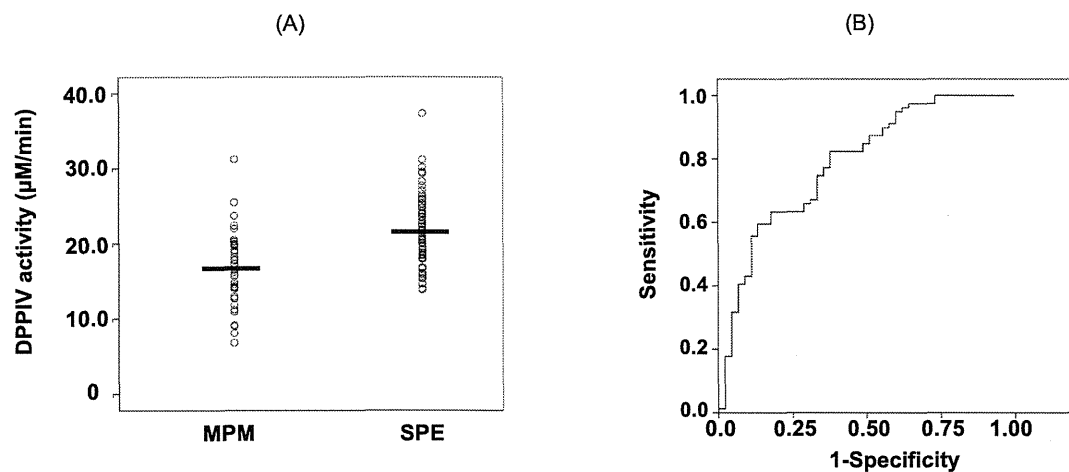
(A)   (B)

Fig. 2. Comparison of serum dipeptidyl peptidase IV (DPPIV) enzyme activity levels. (A) Comparison of serum DPPIV enzyme activity levels in the sera of patients with malignant pleural mesothelioma (MPM) or subjects with past asbestos exposure (SPE). Each dot indicates an individual value and the horizontal bar indicates the median value. (B) Receiver operating curve analysis of serum DPPIV enzyme activity according to the differentiation between patients with MPM and SPE.

value of 17.0 µM/min, the sensitivity was 52.4% and the specificity was 82.3% (Fig. 2B). Taken together, these results indicate that serum DPPIV enzyme activity, as well as serum sCD26 levels, appear to be useful biomarkers for differentiating MPM from the SPE group.

Analysis of Serum sCD26 or DPPIV Activity Among Patients with MPM

As shown above, the serum sCD26 levels and DPPIV enzyme activity appear to be useful biomarkers in patients with MPM. To further clarify the serum sCD26 levels and DPPIV enzyme activity in patients with MPM, we next analyzed the serum sCD26 levels and DPPIV enzyme activity among patients with MPM according to clinical stage. The serum sCD26 levels were significantly decreased in advanced stages (stage III and IV) compared with earlier stages (stage I and II) (P=0.047, Fig. 3A), whereas there was no difference in DPPIV enzyme activity according to the clinical stage of MPM (P=0.333, Fig. 3B). Next, we determined the association between the levels of sCD26 or DPPIV enzyme activity and the OS of patients with MPM. As shown in Fig. 3C, the median OS of patients with MPM who had higher DPPIV enzyme activity (≥17.0 µM/min) was 15.0 months (95% CI, 8.1–21.9 months), which was significantly longer than that of those with lower DPPIV enzyme activity (<17.0 µM/min) who had a median OS of 11.4 months (95% CI, 7.8–15.0 months) (P=0.032, log-rank test). Meanwhile, there was no difference in OS between patients with higher (≥1.00 µg/ml) and lower (<1.00 µg/ml) sCD26 levels (Fig. 3D, P=0.660, log-rank test). These data strongly suggest that serum levels of DPPIV enzyme activity are a predictive biomarker for the prognosis of patients with MPM.

Next, we examined the correlation between DPPIV enzyme activity and sCD26 in serum from patients with MPM. Serum DPPIV enzyme activity was correlated with sCD26 in patients with an epithelioid subtype (r^2=0.770, P=0.000, Fig. 4A), but not in patients with a sarcomatous subtype (r^2=0.089, P=0.835, Fig. 4B).

sCD26 and DPPIV Activity in the Pleural Fluid of Patients with MPM

To further determine the usefulness of sCD26 levels or DPPIV enzyme activity in patients with MPM, we assayed the levels of sCD26 or DPPIV enzyme activity in pleural fluid specimens from patients with MPM. DPPIV enzyme activity in pleural fluid was well correlated with sCD26 in both the epithelioid (r^2=0.895, P=0.000, Fig. 4C) and sarcomatous subtypes (r^2=0.986, P=0.000, Fig. 4D). As shown in Fig. 5A, sCD26 levels in the pleural fluid of MPM patients with an epithelioid subtype were significantly increased compared with the OPD cohort (P=0.012). Moreover, DPPIV enzyme activity in the pleural fluid of MPM patients with an epithelioid subtype was significantly increased compared with that of the OPD cohort (P=0.009, Fig. 5B). These results suggest that sCD26 levels or DPPIV enzyme activity may be good candidates as biomarkers in the pleural fluid of MPM patients with an epithelioid subtype.
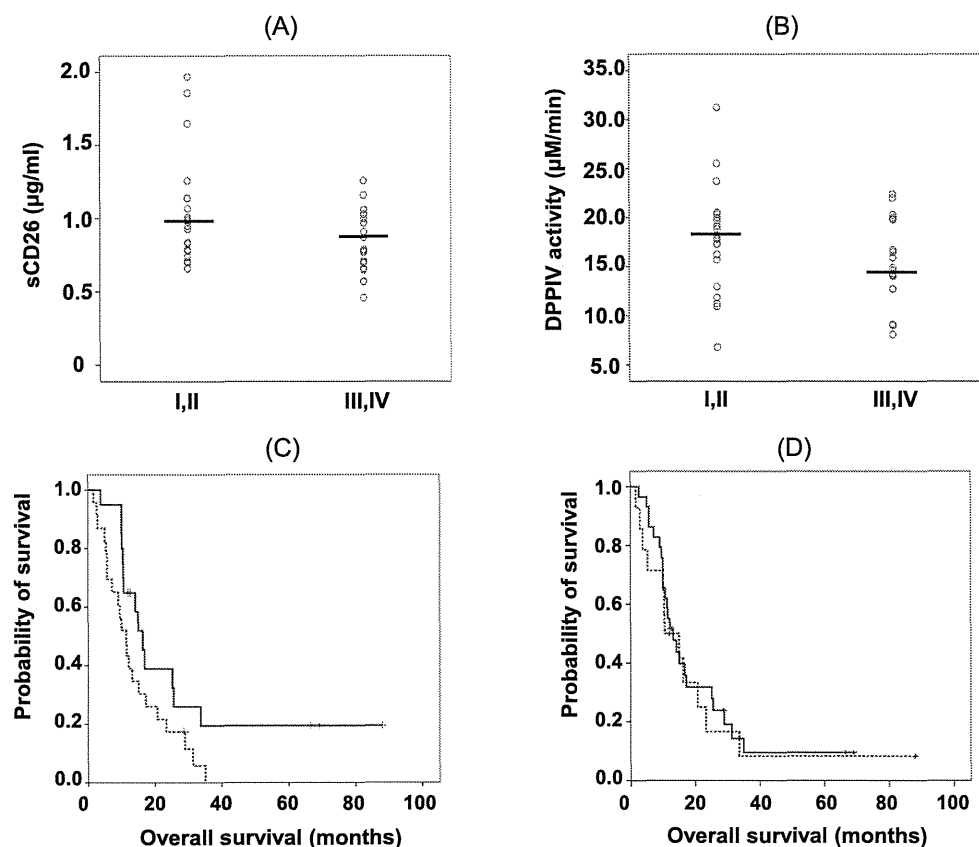
Fig. 3. Serum sCD26 levels or DPPIV enzyme activity according to clinical stage and outcomes. (A) Comparison of levels of serum sCD26 levels and (B) DPPIV enzyme activity among patients with MPM according to clinical stage. The International Mesothelioma Interest Group TNM staging system for MPM was used to determine stage I–IV MPM. The mean values are indicated by horizontal lines. Each dot indicates an individual value and the horizontal bars indicate the median value. (C) Overall survival (OS) in patients with MPM according to those with higher serum sCD26 values (≥1.00 μg/ml, solid line) and lower serum sCD26 values (<1.00 μg/ml, dashed line). (D) OS in patients with MPM according to those with higher (≥17.0 μM/min, solid line) and lower (<17.0 μM/min, dashed line) serum DPPIV enzyme activity.

doi:10.1371/journal.pone.0115647.g003

To further clarify the role of sCD26 levels or DPPIV enzyme activity in pleural fluid, we analyzed the levels of sCD26 or DPPIV enzyme activity in the pleural fluid of MPM patients among patients with each histological subtype. As shown in Fig. 5C, sCD26 levels in the pleural fluid of MPM patients were significantly increased in patients with an epithelioid subtype compared with those with a sarcomatous subtype (P=0.040). In addition, DPPIV enzyme activity in the pleural fluid of MPM patients tended to be increased in patients with an epithelioid subtype compared with those with a sarcomatous subtype (P=0.077). These results suggest that sCD26 levels or DPPIV enzyme activity could be biomarkers in patients with an epithelioid subtype of MPM.

To further determine the possibility that sCD26 levels or DPPIV activity in pleural fluid could act as a biomarker, we analyzed the OS of patients with MPM according to pleural fluid levels of sCD26 or DPPIV enzyme activity. Although we
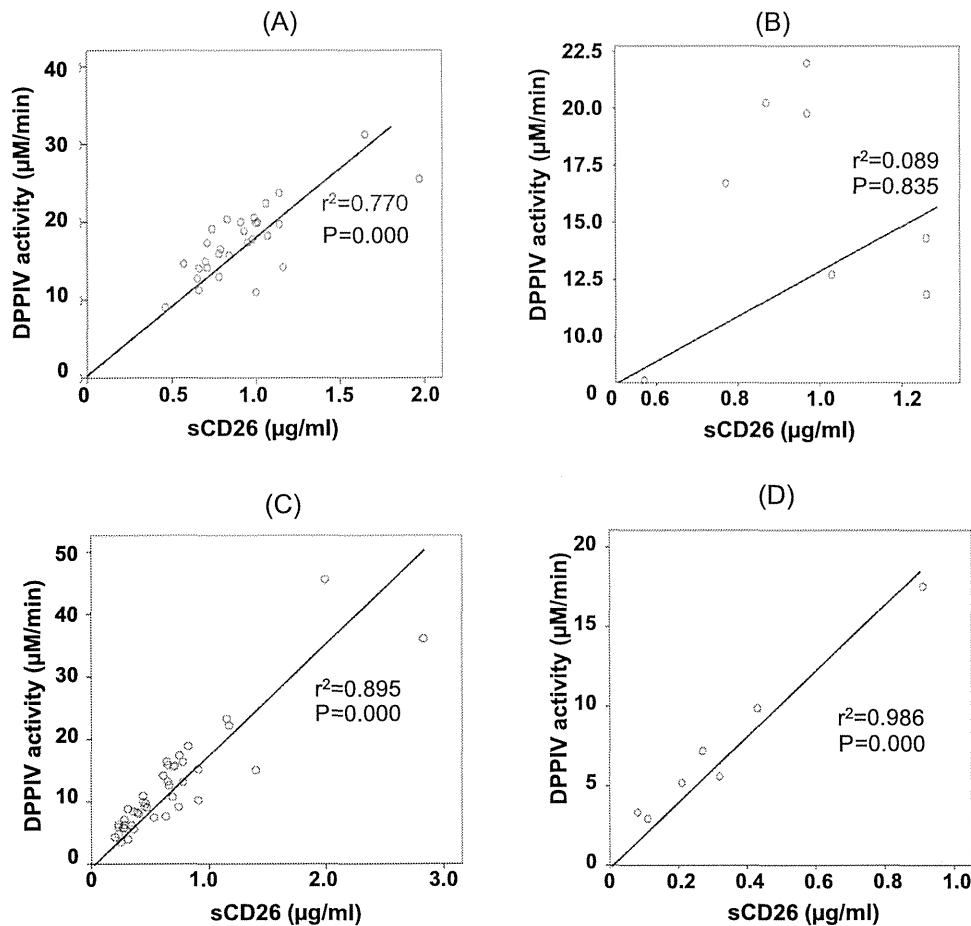
Fig. 4. Correlation between sCD26 levels and DPPIV enzyme activity. Correlation between sCD26 levels and DPPIV enzyme activity in the serum of patients with (A) an epithelioid subtype and (B) sarcomatous subtype of MPM; and in the pleural fluid of patients with (C) an epithelioid subtype and (D) a sarcomatous subtype of MPM.

doi:10.1371/journal.pone.0115647.g004

did not find a significant difference in OS according to sCD26 levels (P=0.260) or DPPIV enzyme activity (P=0.582) (Fig. 6A or B, respectively), patients with MPM who had a lower specific DPPIV activity, determined as DPPIV/sCD26 (<21.0 nmol/min/mg sCD26), had significantly prolonged survival compared with those with higher specific DPPIV activity (≥21.0 nmol/min/mg sCD26) (median OS: 18.5 months vs 12.2 months, P=0.028 by log-rank test) (Fig. 6C). Taken together with the above data, our results strongly suggest that DPPIV activity in serum or pleural fluid may be a useful biomarker predictive of the prognosis of MPM patients.

Serum and pleural fluid SMRP

To make a comparative review of the usefulness of sCD26, we determined serum and pleural fluid SMRP. Median values of serum and pleural fluid SMRP in MPM
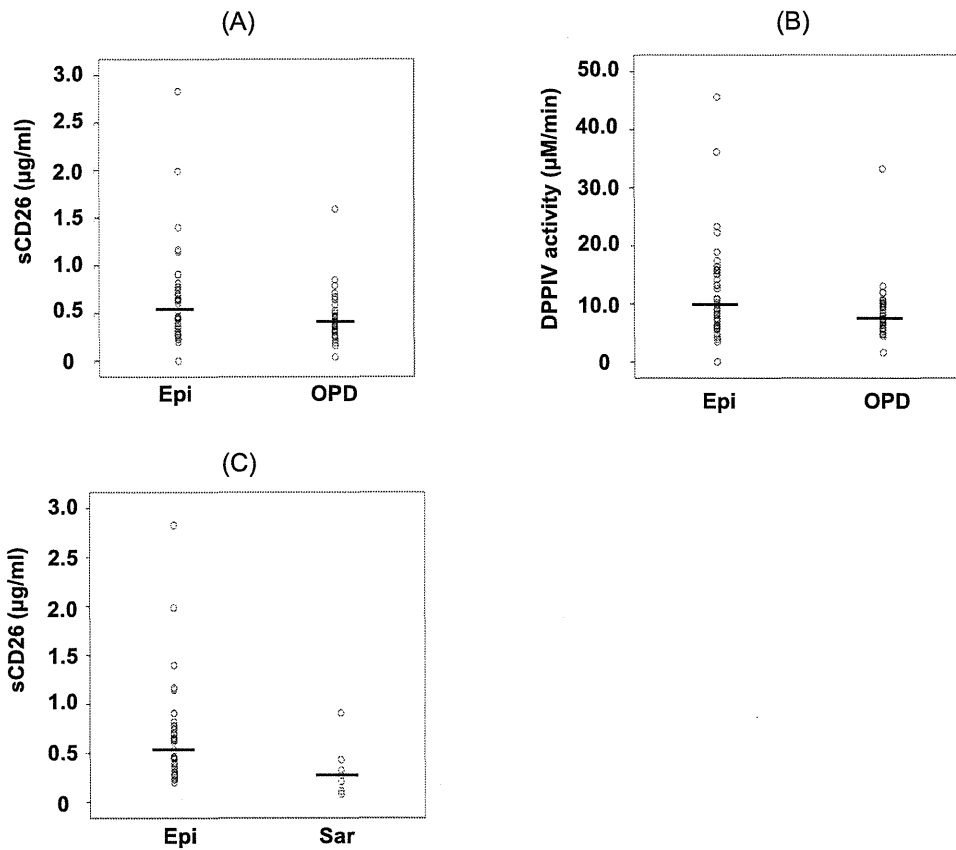
Fig. 5. The levels of sCD26 or DPPIV enzyme activity in pleural fluid. (A) sCD26 levels and (B) DPPIV enzyme activity in the pleural fluid of patients with an epithelioid subtype of MPM (Epi) or with other pleural diseases (OPD). (C) sCD26 levels in the pleural fluid of patients with an Epi or sarcomatous (Sar) subtype of MPM. Each dot indicates an individual value and the horizontal bars indicate the median value.

patients were 0.43 and 15.37 mmol/l, respectively. Median value of pleural fluid SMRP in epithelioid MPM was 17.28 mmol/l. Median values of serum SMRP in SPE and pleural fluid SMRP in OPD were 0.90 and 0.43 mmol/l, respectively. Pleural fluid SMRP in MPM was significantly higher than in OPD (P=0.000) and serum SMRP in MPM was significantly higher than in SPE (P=0.000). To further clarify the usefulness of serum SMRP for differentiating MPM from SPE, we performed a ROC analysis. The AUC value for the differential diagnosis between these 2 groups was 0.738 (95% CI, 0.638–0.838) (data not shown).

Discussion

We examined the usefulness of serum and pleural fluid sCD26 levels and DPPIV enzyme activity as clinical biomarkers of MPM. Serum sCD26 level and DPPIV enzyme activity were significantly decreased in patients with MPM compared with the SPE group. Generally, negative biomarkers have been difficult as markers of
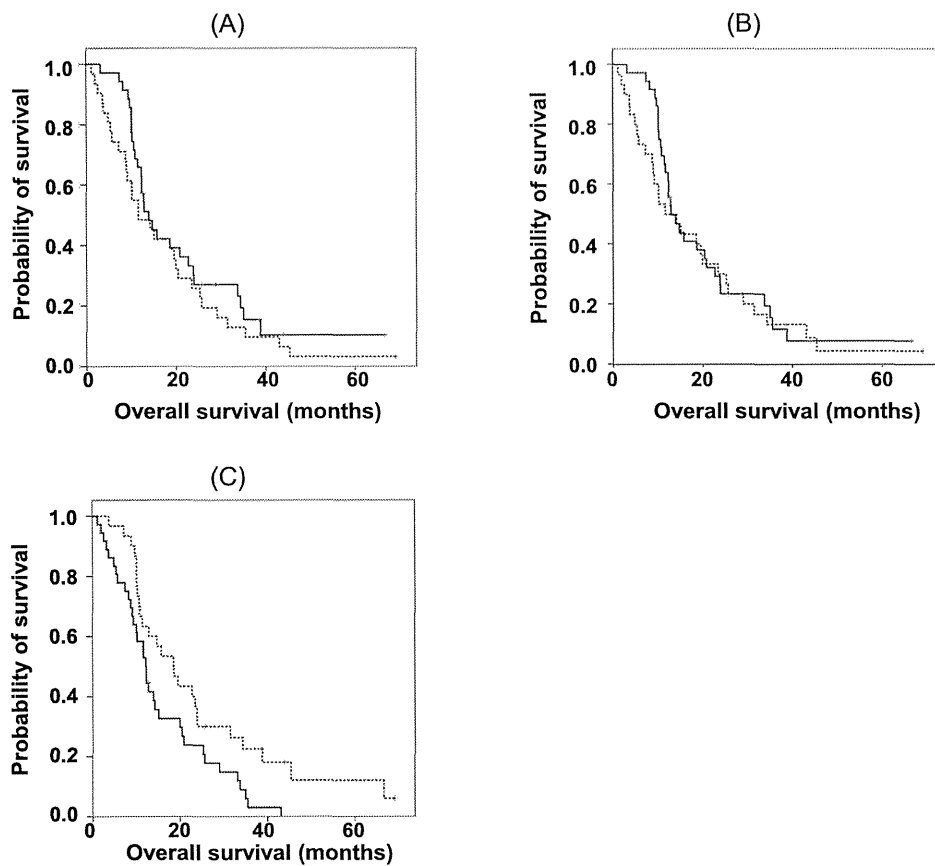
Fig. 6. Overall survival in patients with malignant pleural mesothelioma according to soluble sCD26 levels. OS according to those with (A) higher (≥0.45 μg/ml, solid line) and lower (<0.45 μg/ml, dashed line) pleural fluid soluble sCD26 (sCD26) values; (B) higher (≥9.0 μM/min, solid line) and lower (<9.0 μM/min, dashed line) pleural fluid DPPIV enzyme activity; and (C) a higher (≥21.0, solid line) and lower (<21.0, dashed line) fraction of DPPIV/sCD26 in the pleural fluid.

doi:10.1371/journal.pone.0115647.g006

clinical significance. However, these results indicate the usefulness of these markers for early detection of MPM among the SPE group. Our results indicate that sCD26 could be compared favorably with SMRP, which is one of the most promising molecular biomarker of MPM at this time. In addition, sCD26 and DPPIV enzyme activity in pleural fluid was increased in patients with an epithelial subtype of MPM, and higher than those with OPD. These results indicate the clinical significance of sCD26 levels and DPPIV enzyme activity in pleural fluid as a diagnostic marker of the epithelial subtype of MPM. Furthermore, survival analyses demonstrated that serum DPPIV enzyme activity and specific DPPIV enzyme activity, determined as DPPIV/sCD26 in pleural fluid, could be a prognostic factor in patients with MPM.

MPM cases are usually diagnosed at an advanced stage and show poor response to treatment, so it is important to establish a molecular biomarker that can help diagnose MPM at earlier stages. In addition, focus should be put on screening

high-risk subjects because most cases of MPM develop among those with an occupational or environmental history of past asbestos exposure. In this regard, some previous reports of molecular diagnostic markers for MPM exist. Robinson et al reported that serum SMRP was higher in patients with MPM compared with those with other cancers or other inflammatory lung or pleural diseases [24]. They also reported that 7 of 40 asbestos-exposed individuals had elevated serum concentrations of SMRP, and 3 of those 7 developed MPM within 5 years [24]. Scherpereel et al also reported that the serum SMRP level was higher in patients with MPM than in patients with pleural metastasis or benign pleural diseases [6]. Pass et al reported that serum osteopontin was higher in patients with MPM than in subjects with a history of asbestos exposure [25]. Recently, Shiomi et al reported that serum N-ERC/mesothelin levels were higher in patients with MPM compared with those with other diseases, including asbestos-related nonmalignant diseases [8]. In these previous reports, the definition of the control group was ambiguous; some included healthy subjects with a history of asbestos exposure, whereas others included patients with other asbestos-related benign diseases such as asbestosis. In the current study, serum sCD26 levels in patients with MPM were compared with those with past asbestos exposure and pleural plaques.

Pleural plaques are discrete, white to yellow-white, irregularly shaped, frequently calcified, and raised structures involving the parietal pleura [26]. They are not included in asbestos-related pleural diseases, but are established as a medical indicator of past asbestos exposure. Future studies are warranted to compare the utility of these markers for the differential diagnosis of MPM with a unified control group. In addition, the combination of these markers should be examined for a more accurate differential diagnosis.

There are some previous reports concerning the significance of CD26 levels in malignant conditions. Previous studies of CD26 have yielded varying results in different cancers. Preclinical studies show that increased CD26 expression inhibited metastasis in ovarian cancer [27], whereas suppression of CD26 promoted metastasis in prostate cancer [28]. On the other hand, inhibition of CD26 in renal cell carcinoma decreased tumor growth and reduced the ability of cancer cells to bind to fibronectin and collagen [17]. Moreover, clinical studies in thyroid cancer, gastrointestinal stromal tumors, and T cell non-Hodgkin's lymphoma/leukemias suggested that CD26 expression was associated with distant metastasis, recurrence after resection, or poor survival [29–31]. The multiple functions of CD26 may account for its various roles in different cancers [32]. Our recent study showed that CD26 expression in mesothelioma cells was associated with enhanced proliferative activity [19], and that CD26-positive mesothelioma cell lines appeared to have the characteristics of cancer stem cells [33].

The current study demonstrated that serum sCD26 levels were decreased in patients with MPM. Previously, Cordero et al reported that serum sCD26 was significantly lower in patients with colorectal cancer compared with healthy donors [34]. Their results are similar to those in the current study in terms of serum sCD26 levels being lower in cancer patients. As Cordero described, these findings indicate that the drop in sCD26 levels are related to an impaired immune

system. These speculations are supported by data showing that CD26 and DPPIV activity are critical in T-cell biology as markers of T-cell activation. In addition, our current study demonstrated that serum sCD26 levels were decreased in advanced stages of MPM. Based on these findings, serum sCD26 levels might reflect impaired immune functions during the development and progression of MPM. Alternatively, there is another recent perception that serum DPPIV activity is one of the so-called adipokines, which are produced and released from adipose tissue [35, 36]. These adipokines are increased in obesity and reduced after weight loss, and are potential biomarkers of metabolic syndrome [37]. The relationship between decreased sCD26 and weight loss due to the development or progression of MPM should be clarified in future investigations.

In the current study, we also determined the sCD26 levels in the pleural fluid of patients with MPM and showed that sCD26 levels were higher in patients with an epithelioid subtype of MPM compared with those with a sarcomatous subtype. In a recent report, we demonstrated that CD26 expression in the tumor was higher in the epithelioid subtype of MPM than in other subtypes [19]. Based on these results, we suggest that sCD26 levels in the pleural fluid is secreted or released from MPM cells in the thorax. All these findings indicate that sCD26 in the serum and pleural fluid is released by different mechanisms. These findings are quite interesting in terms of the significance of CD26 levels in patients with MPM, in addition to the clinical usefulness of sCD26 as a molecular biomarker.

In previous reports, DPPIV enzyme activity was correlated with sCD26 concentration in healthy subjects and patients with type II diabetes [23, 38]. In the current study, the correlation was shown in patients with MPM except for those with the sarcomatous subtype (Fig. 4B). Although we cannot exclude the possibility that any genetic mutations or epigenetic modifications in CD26 appear to occur and potentiate DPPIV enzyme activity in the sarcomatous subtype of MPM, the discrepancy of specific DPPIV enzyme activity in the serum of patients with a sarcomatous subtype of MPM will be investigated in future studies.

In conclusion, we demonstrate the clinical significance of sCD26 levels and DPPIV activity in the sera and pleural fluid of patients with MPM. Serum sCD26 levels or DPPIV enzyme activity might be useful as early diagnostic markers or prognostic markers in patients with MPM, or as a screening tool to detect those at high-risk for development of MPM among SPE. The sCD26 levels in pleural fluid could be a useful diagnostic marker of the epithelioid subtype of MPM. Further validation studies are essential to clarify the clinical usefulness of sCD26 levels in patients with MPM.

Supporting Information

S1 Table. Soluble CD26 (sCD26) levels and dipeptidyl peptidase IV (DPPIV) enzyme activity values. The median and interquartile range values of the serum and pleural fluid sCD26 levels and DPPIV enzyme activity are shown.
doi:10.1371/journal.pone.0115647.s001 (DOCX)

Acknowledgments

Author Contributions

References

1. Ismail-Khan R, Robinson LA, Williams CC Jr, Garrett CR, Bepler G, et al. (2006) Malignant pleural mesothelioma: a comprehensive review. Cancer Control 13: 255–263.

2. Gemba K, Fujimoto N, Kato K, Aoe K, Takeshima Y, et al. (2012) National survey of malignant mesothelioma and asbestos exposure in Japan. Cancer Sci 103: 483–490.

3. Gemba K, Fujimoto N, Aoe K, Kato K, Takeshima Y, et al. (2013) Treatment and survival analyses of malignant mesothelioma in Japan. Acta Oncol 52: 803–808.

4. Robinson BW, Lake RA (2005) Advances in malignant mesothelioma. N Engl J Med 353: 1591–1603.

5. Britton M (2002) The epidemiology of mesothelioma. Semin Oncol 29: 18–25.

6. Scherpereel A, Grigoriu B, Conti M, Gey T, Gregoire M, et al. (2006) Soluble mesothelin-related peptides in the diagnosis of malignant pleural mesothelioma. Am J Respir Crit Care Med 173: 1155–1160.

7. Fujimoto N, Gemba K, Asano M, Wada S, Ono K, et al. (2010) Soluble mesothelin-related protein in pleural effusion from patients with malignant pleural mesothelioma. Exp Ther Med 1: 313–317.

8. Shiomi K, Hagiwara Y, Sonoue K, Segawa T, Miyashita K, et al. (2008) Sensitive and specific new enzyme-linked immunosorbent assay for N-ERC/mesothelin increases its potential as a useful serum tumor marker for mesothelioma. Clin Cancer Res 14: 1431–1437.

9. Pass HI, Levin SM, Harbut MR, Melamed J, Chiriboga L, et al. (2012) Fibulin-3 as a blood and effusion biomarker for pleural mesothelioma. N Engl J Med 367: 1417–1427.

10. Morimoto C, Schlossman SF (1998) The structure and function of CD26 in the T-cell immune response. Immunol Rev 161: 55–70.

11. Kameoka J, Tanaka T, Nojima Y, Schlossman SF, Morimoto C (1993) Direct association of adenosine deaminase with a T cell activation antigen, CD26. Science 261: 466–469.

12. Ishii T, Ohnuma K, Murakami A, Takasawa N, Kobayashi S, et al. (2001) CD26-mediated signaling for T cell activation occurs in lipid rafts through its association with CD45RO. Proc Natl Acad Sci U S A 98: 12138–12143.

13. Ohnuma K, Yamochi T, Uchiyama M, Nishibashi K, Yoshikawa N, et al. (2004) CD26 up-regulates expression of CD86 on antigen-presenting cells by means of caveolin-1. Proc Natl Acad Sci U S A 101: 14186–14191.

14. Carbone A, Gloghini A, Zagonel V, Aldinucci D, Gattei V, et al. (1995) The expression of CD26 and CD40 ligand is mutually exclusive in human T-cell non-Hodgkin's lymphomas/leukemias. Blood 86: 4617–4626.

15. Droz D, Zachar D, Charbit L, Gogusev J, Chretein Y, et al. (1990) Expression of the human nephron differentiation molecules in renal cell carcinomas. Am J Pathol 137: 895–905.

16. Stange T, Kettmann U, Holzhausen HJ (2000) Immunoelectron microscopic demonstration of the membrane proteases aminopeptidase N/CD13 and dipeptidyl peptidase IV/CD26 in normal and neoplastic renal parenchymal tissues and cells. Eur J Histochem 44: 157–164.

17. Inamoto T, Yamochi T, Ohnuma K, Iwata S, Kina S, et al. (2006) Anti-CD26 monoclonal antibody-mediated G1-S arrest of human renal clear cell carcinoma Caki-2 is associated with retinoblastoma

substrate dephosphorylation, cyclin-dependent kinase 2 reduction, p27(kip1) enhancement, and disruption of binding to the extracellular matrix. Clin Cancer Res 12: 3470–3477.

18. **Inamoto T, Yamada T, Ohnuma K, Kina S, Takahashi N, et al.** (2007) Humanized anti-CD26 monoclonal antibody as a treatment for malignant mesothelioma tumors. Clin Cancer Res 13: 4191–4200.

19. **Aoe K, Amatya VJ, Fujimoto N, Ohnuma K, Hosono O, et al.** (2012) CD26 overexpression is associated with prolonged survival and enhanced chemosensitivity in malignant pleural mesothelioma. Clin Cancer Res 18: 1447–1456.

20. **Churg A, Inai K, Samet J** (2004) Tumours of the pleura. Travis W, Brambilla E, Muller-Hermelink H, Harris C, editors. Lyon, France: IARC Press.

21. **Rusch VW** (1995) A proposed new international TNM staging system for malignant pleural mesothelioma. From the International Mesothelioma Interest Group. Chest 108: 1122–1128.

22. **Fujimoto N, Gemba K, Asano M, Fuchimoto Y, Wada S, et al.** (2013) Hyaluronic acid in the pleural fluid of patients with malignant pleural mesothelioma. Respir Investig 51: 92–97.

23. **Ohnuma K, Saito T, Hatano R, Hosono O, Iwata S, et al.** (2014) Comparison of Two Commercial ELISAs against an In-House ELISA for Measuring Soluble CD26 in Human Serum. J Clin Lab Anal, DOI: 10.1002/jcla.21736

24. **Robinson BW, Creaney J, Lake R, Nowak A, Musk AW, et al.** (2003) Mesothelin-family proteins and diagnosis of mesothelioma. Lancet 362: 1612–1616.

25. **Pass HI, Lott D, Lonardo F, Harbut M, Liu Z, et al.** (2005) Asbestos exposure, pleural mesothelioma, and serum osteopontin levels. N Engl J Med 353: 1564–1573.

26. **Hammar SP** (2011) Asbestos-Induced Pleural Disease. Dodson RF, Hammar SP, editors. Boca Raton: CRC Press.

27. **Kajiyama H, Kikkawa F, Suzuki T, Shibata K, Ino K, et al.** (2002) Prolonged survival and decreased invasive activity attributable to dipeptidyl peptidase IV overexpression in ovarian carcinoma. Cancer Res 62: 2753–2757.

28. **Sun YX, Pedersen EA, Shiozawa Y, Havens AM, Jung Y, et al.** (2008) CD26/dipeptidyl peptidase IV regulates prostate cancer metastasis by degrading SDF-1/CXCL12. Clin Exp Metastasis 25: 765–776.

29. **Yamaguchi U, Nakayama R, Honda K, Ichikawa H, Hasegawa T, et al.** (2008) Distinct gene expression-defined classes of gastrointestinal stromal tumor. J Clin Oncol 26: 4100–4108.

30. **Ho L, Aytac U, Stephens LC, Ohnuma K, Mills GB, et al.** (2001) In vitro and in vivo antitumor effect of the anti-CD26 monoclonal antibody 1F7 on human CD30+ anaplastic large cell T-cell lymphoma Karpas 299. Clin Cancer Res 7: 2031–2040.

31. **Hirai K, Kotani T, Aratake Y, Ohtaki S, Kuma K** (1999) Dipeptidyl peptidase IV (DPP IV/CD26) staining predicts distant metastasis of 'benign' thyroid tumor. Pathol Int 49: 264–265.

32. **Havre PA, Abe M, Urasaki Y, Ohnuma K, Morimoto C, et al.** (2008) The role of CD26/dipeptidyl peptidase IV in cancer. Front Biosci 13: 1634–1645.

33. **Ghani FI, Yamazaki H, Iwata S, Okamoto T, Aoe K, et al.** (2011) Identification of cancer stem cell markers in human malignant mesothelioma cells. Biochem Biophys Res Commun 404: 735–742.

34. **Cordero OJ, Ayude D, Nogueira M, Rodriguez-Berrocal FJ, de la Cadena MP** (2000) Preoperative serum CD26 levels: diagnostic efficiency and predictive value for colorectal cancer. Br J Cancer 83: 1139–1146.

35. **Kershaw EE, Flier JS** (2004) Adipose tissue as an endocrine organ. J Clin Endocrinol Metab 89: 2548–56.

36. **Ahima RS, Osei SY** (2008) Adipokines in obesity. Front Horm Res 36: 182–97.

37. **Lamers D, Famulla S, Wronkowitz N, Hartwig S, Lehr S, et al.** (2011) Dipeptidyl peptidase 4 is a novel adipokine potentially linking obesity to the metabolic syndrome. Diabetes 60: 1917–1925.

38. **Andrieu T, Thibault V, Malet I, Laporte J, Bauvois B, et al.** (2003) Similar increased serum dipeptidyl peptidase IV activity in chronic hepatitis C and other viral infections. J Clin Virol 27: 59–68.

Blockade of CD26 Signaling Inhibits Human Osteoclast Development

Hiroko Nishida,[1] Hiroshi Suzuki,[1] Hiroko Madokoro,[1] Mutsumi Hayashi,[1] Chikao Morimoto,[2] Michiie Sakamoto,[1] and Taketo Yamada[1]

[1]Department of Pathology, Keio University School of Medicine, Shinjuku-ku, Tokyo, Japan
[2]Department of Therapy Development and Innovation for Immune Disorders and Cancers, Graduate School of Medicine, Juntendo University, Bunkyo-ku, Tokyo, Japan

ABSTRACT

Bone remodeling is maintained by the delicate balance between osteoblasts (OBs) and osteoclasts (OCs). However, the role of CD26 in regulating bone remodeling has not yet been characterized. We herein show that CD26 is preferentially expressed on normal human OCs and is intensely expressed on activated human OCs in osteolytic bone alterations. Macrophage-colony stimulating factor (M-CSF) and soluble receptor activator of NF-κB ligand (sRANKL) induced human OC differentiation, in association with CD26 expression on monocyte-macrophage lineage cells. CD26 expression was accompanied by increased phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK), which is crucial for early human OC differentiation. The humanized anti-CD26 monoclonal antibody, huCD26mAb, impaired the formation and function of tartrate-resistant acid phosphatase (TRAP)/CD26 positive multi-nucleated (nuclei > 3) OCs with maturation in the manner of dose-dependency. It was revealed that huCD26mAb inhibits early OC differentiation via the inactivation of MKK3/6, p38 MAPK and subsequent dephosphorylation of microphthalmia-associated transcription factor (mi/Mitf). These inhibitions occur immediately after RANKL binds to RANK on the human OC precursor cells and were demonstrated using the OC functional assays. huCD26mAb subsequently impaired OC maturation and bone resorption by suppressing the expression of TRAP and OC fusion proteins. In addition, p38 MAPK inhibitor also strongly inhibited OC formation and function. Our results suggest that the blockade of CD26 signaling impairs the development of human functional OCs by inhibiting p38 MAPK-mi/Mitf phosphorylation pathway and that targeting human OCs with huCD26mAb may have therapeutic potential for the treatment of osteolytic lesions following metastasis to alleviate bone destruction and reduce total skeletal-related events (SREs). © 2014 American Society for Bone and Mineral Research.

KEY WORDS: CD26; OSTEOCLAST; P38 MAPK; OSTEOCLAST PRECURSOR; BONE RESORPTION

Introduction

Bone mass is maintained by the delicate balance between osteoblastic bone formation and osteoclastic bone resorption, a process called bone remodeling.[1,2] Osteoclasts (OCs) are unique bone-resorbing multi-nuclear giant cells formed by the fusion of OC precursor cells, which are derived from hematopoietic cells of the monocyte-macrophage lineage under the control of the bone marrow microenvironment. Several cytokines are essential for OC development, in particular, macrophage colony stimulating factor (M-CSF) and soluble receptor activator of NF-κB ligand (sRANKL). sRANKL is a transmembrane glycoprotein belonging to the tumor necrosis factor (TNF-α) superfamily and for years osteoblastic cells on bone surface had been considered to supply sRANKL responsible for OC formation.[1–4] However, recent observations suggested that osteocytes serve as the major source of sRANKL in regulating OC formation.[5,6] OC

precursor cells express RANK, the receptor for RANKL, and the OC differentiation is dependent on RANKL. Activation of RANK on the surface of OC precursor cells by its ligand, RANKL, leads to the activation of downstream signaling pathways, including SAPK/JNK, p38MAPK, ERK, and IKKβ pathways, leading to the expression of osteoclastgenesis-specific genes, such as encoding tartrate-resistant acid phosphatase (TRAP), Cathepsin K, αv Integrin and Src. Finally, RANKL also induces the secretion of lytic enzymes into a sealed resorption vacuole formed between the basal surface of the OCs and the bone surface. The secretion of these enzymes lead to the activation of TRAP and Cathepsin K, which are the main enzymes responsible for the degradation of the bone mineral and collagen matrix, and they are essential for OC maturation.[1–4]

Elevated OC formation and function have been observed in patients with osteolytic bone lesions following metastasis, including patients with prostate cancer, breast cancer, lung