Ⅲ.　研究成果の別刷

Chapter

THE USE OF THE HUMANIZED ANTI-CD26 MONOCLONAL ANTIBODY YS110 AS A NOVEL TARGETED THERAPY FOR REFRACTORY CANCERS AND IMMUNE DISORDERS

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ABSTRACT

CD26 is a 110 kDa surface glycoprotein with intrinsic dipeptidyl peptidase IV (DPPIV) activity that is expressed on numerous cell types and has a multitude of biological functions. We have had a long-standing interest in the role of CD26 in cancer biology and immune regulation and developed YS110, a humanized monoclonal antibody (mAb) with high affinity to the CD26 antigen. Our group has shown that *in vivo* administration of YS110 inhibits tumor cell growth, migration and invasion, and enhances survival of mouse xenograft models inoculated with malignant mesothelioma (MPM), renal cell carcinoma, non-small-cell lung carcinoma, ovarian carcinoma or T-cell lymphoma via multiple mechanisms of action. The first-in-human (FIH) phase 1 clinical trial of this mAb for CD26-expressing solid tumors, particularly refractory MPM, was conducted from 2009 to 2014, with results being recently published. Our FIH study demonstrates that YS110 therapy exhibits a favorable safety profile and results in encouraging disease stabilization in a number of patients with advanced/refractory CD26-expressing cancers. In addition, we have robust evidence through multiple studies that CD26 functions as a T-cell costimulatory molecule and has an important role in T-cell biology. High CD26 cell surface expression is correlated with enhanced migratory ability through endothelial cells, and $CD26^+$ T cells are present at high levels in the inflammatory site of graft-versus-host disease (GVHD) and various autoimmune diseases such as rheumatoid arthritis, multiple sclerosis and Graves' disease. Our recent work with a xenogeneic GVHD murine model also demonstrates that administration of YS110 decreases GVHD severity and prolongs survival, while preserving the graft-versus-leukemia effect. Furthermore, recent work has shown that infection by the novel Middle East respiratory syndrome coronavirus (MERS-CoV) is mediated by the use of CD26 as a functional receptor. We have identified the domains of CD26 involved in the binding of MERS-CoV and showed that YS110 treatment significantly inhibits viral infection. We herein review novel findings strongly suggesting that YS110 represents a promising novel therapy for refractory cancers, immune disorders and MERS-CoV infection.

Keywords: CD26/DPPIV, YS110, malignant mesothelioma, immune disorders, MERS-CoV

1. INTRODUCTION

CD26 is a 110-kDa, type II transmembrane glycoprotein with known dipeptidyl peptidase IV (DPPIV, EC 3.4.14.5) activity in its extracellular domain and is capable of cleaving N-terminal dipeptides with either L-proline or L-alanine at the penultimate position (Ohnuma et al., 2008a). CD26 activity is dependent on cell type and the microenvironment factors that can influence its multiple biological roles (Ohnuma et al., 2011; Thompson et al., 2007). CD26 is expressed on various tumors including malignant pleural mesothelioma (MPM), renal cell carcinoma (RCC), lung cancer, colorectal cancer (CRC), hepatocellular carcinoma, prostate cancer, gastrointestinal stromal tumor (GIST), thyroid carcinoma, and hematologic malignancies such as T-anaplastic large cell lymphoma, T-lymphoblastic lymphoma and T-acute lymphoblastic leukemia (Havre et al., 2008; Ohnuma and Morimoto, 2013; Thompson et al., 2007). We have had a long-standing interest in the role of CD26 in cancer biology and developed YS110, a humanized monoclonal antibody (mAb) with high affinity to the CD26 antigen. Our group has shown that *in vitro* and *in vivo* administration of YS110 inhibited tumor cell growth, migration and invasion, and enhanced survival of mouse xenograft models inoculated with MPM, RCC, non-small-cell lung carcinoma, ovarian carcinoma or T-cell lymphoma via multiple mechanisms of action (Inamoto et al., 2007; Inamoto et al., 2006; Ho et al., 2001). The first-in-human (FIH) phase 1 clinical trial of this mAb for CD26-expressing solid tumors, particularly refractory MPM, was conducted with results being recently published (Angevin et al., 2017). Our FIH study demonstrated that YS110 therapy exhibits a favorable safety profile and resulted in encouraging disease stabilization in a number of patients with advanced/refractory CD26-expressing cancers.

In addition, we have robust evidence through multiple studies that CD26 functions as a T-cell costimulatory molecule and has an important role in T-cell biology and overall immune function (Morimoto and Schlossman, 1998; Ohnuma et al., 2008a). We identified caveolin-1 as a costimulatory ligand for CD26 in T cells, and showed that CD26-caveolin-1 interaction led to activation of both CD4 T cells and antigen presenting cells (APCs) (Ohnuma et al., 2004; Ohnuma et al., 2005; Ohnuma et al., 2007). Moreover, High CD26 cell surface expression was correlated with enhanced migratory ability through endothelial cells (Masuyama et al., 1992), and $CD26^+$ T cells were present at high levels in the inflammatory site of graft-versus-host disease (GVHD) and various autoimmune diseases such as rheumatoid arthritis (RA), multiple sclerosis and Graves' disease (Hatano et al., 2013a; Ohnuma et al., 2015a; Ohnuma et al., 2011). Our recent work with a xenogeneic GVHD murine model also demonstrated that administration of YS110 decreased GVHD severity and prolonged survival, while preserving the graft-versus-leukemia (GVL) effect (Hatano et al., 2013a).

Furthermore, recent work has shown that infection by the Middle East respiratory syndrome coronavirus (MERS-CoV) is mediated by the use of CD26 as a functional receptor (Raj et al., 2013). MERS-CoV is a novel coronavirus identified in patients with severe lower respiratory tract infections with almost 50% of cases resulting in lethal lower respiratory tract infections (Zaki et al., 2012; Enserink, 2013). We have identified the domains of CD26 involved in the binding of MERS-CoV and showed that YS110 treatment significantly inhibited viral infection (Ohnuma et al., 2013). We recently reviewed our significant findings and the early clinical development of a CD26-targeted therapy for MPM (Ohnuma et al., 2017). We herein review novel findings strongly suggesting that YS110 represents a promising novel therapy not only for MPM but also for other refractory cancers, immune disorders and MERS-CoV infection.

2. CANCERS

2.1. Malignant Pleural Mesothelioma

Our recent in-depth studies of CD26 expression in MPM revealed that CD26 is preferentially expressed in MPM cells but not in normal mesothelial cells (Amatya et al., 2011; Aoe et al., 2012). Our immunohistochemical analysis showed that membranous expression of CD26 was particularly found in epithelioid mesotheliomas, but not in sarcomatoid mesotheliomas (Aoe et al., 2012). In addition, we identified SP (side-population), CD9, CD24, and CD26 as MPM cancer stem cell markers that correlated with primary stem cell signatures (Ghani et al., 2011; Yamazaki et al., 2012). These intriguing findings propelled our development of CD26-targeted therapy for MPM. For this purpose, we had developed a novel humanized anti-CD26 mAb, YS110. We recently reviewed the functional role of CD26 in the neoplastic biology of MPM and our robust *in vitro* and *in vivo* data investigating the anti-tumor effect of YS110 on MPM (Ohnuma et al., 2017). We conducted the FIH phase 1 clinical trial of YS110 for patients with refractory MPM and other CD26-positive solid tumors (Angevin et al., 2017). Thirty-three heavily pre-treated patients with CD26-positive cancers including 22 MPM underwent YS110 administration. Our FIH study conclusively demonstrated that YS110 exhibits a favorable safety profile and substantial clinical activity in heavily pre-treated CD26-positive MPM patients who had previously progressed on conventional standard chemotherapies. Further clinical trial of YS110 for MPM is in progress worldwide (ClinicalTrials.gov, 2017).

2.2. Other Cancers

In contrast to our robust findings regarding the role of CD26/DPPIV on MPM (Ohnuma et al., 2017), the exact role of CD26/DPPIV in other cancers remains to be elucidated, partly due to its variable expression on these tumors. In general, it is strongly expressed on some cancers, while

being absent or present at low levels in others (Thompson et al., 2007). Furthermore, given its multiple biological functions, including its ability to associate with several key proteins and its cleavage of a number of soluble factors to regulate their function, it is likely that the CD26/DPPIV effect on tumor biology is at least partly mediated by the effect of these biological functions on specific tumor types (Havre et al., 2008).

2.2.1. Renal Cancer

CD26 has also been shown to be expressed on RCC (Stange et al., 2000; Inamoto et al., 2006; Hatano et al., 2014), including the cell lines Caki-1, Caki-2, VMRC-RCW, and ACHN (Inamoto et al., 2006). We showed that anti-CD26 mAb inhibition of the Caki-2 cell line was associated with G1/S cell cycle arrest, enhanced $p27^{kip1}$ expression, downregulation of cyclin-dependent kinase 2 (CDK2) and dephosphorylation of retinoblastoma substrate (Rb) (Inamoto et al., 2006). We also found that anti-CD26 mAb therapy attenuated Akt activity (Figure 1D) and internalized cell surface CD26 leading to decreased CD26 binding to collagen and fibronectin (Figure 1A and 1B). Treatment with anti-CD26 murine mAb inhibited the growth of human RCC and significantly enhanced survival in a mouse xenograft model (Figure 1C). Our FIH phase 1 study demonstrated that disease stabilization was observed in heavily pre-treated CD26-positive MPM and RCC patients who had previously progressed on conventional standard therapies (Angevin et al., 2017), suggesting that YS110 treatment may have potential clinical use for CD26-positive RCC.

2.2.2. Lung Cancer

CD26 expression in lung cancer appears to be dependent on the specific histologic subtype. Liu et al., showed that CD26 was highly expressed in poorly differentiated lung adenocarcinomas compared to highly differentiated lung adenocarcinomas utilizing human lung adenocarcinoma tissue microarrays (Liu et al., 2013). These investigators demonstrated that CD26 inhibition by shRNA significantly decreased the invasive and migratory capacity of human lung adenocarcinoma cell line,

SPC-A-1sci cells, while CD26 overexpression increased the invasive and migratory capacity of SPC-A-1 cells (Liu et al., 2013). We are currently investigating *in vitro* and *in vivo* anti-tumor activity of YS110 on lung cancers, and our preliminary data show that treatment with YS110 inhibits the growth of human lung cancer cell lines *in vitro* and *in vivo*. Definitive data will be presented in the near future.

2.2.3. Colorectal Cancer

Pang et al., identified a subpopulation of $CD26^+$ cells uniformly presenting in both primary and metastatic tumors in colorectal cancer, and showed that $CD26^+$ cancer cells are associated with enhanced invasiveness and chemoresistance (Pang et al., 2010). These investigators showed that in $CD26^+$ colorectal cancer cells, mediators of epithelial to mesenchymal transition (EMT) contributed to the invasive phenotype and metastatic capacity. These results suggested that $CD26^+$ cells are cancer stem cells in colorectal cancer, and that CD26/DPPIV can be targeted for metastatic colorectal cancer therapy. More recently, significantly higher CD26 expression has been shown to be correlated with poorly differentiated tumor, late tumor node metastasis (TNM) stage (TNM stage III and stage IV), and development of metastasis (Lam et al., 2014). Moreover, a high CD26 expression level is a predictor of poor outcome after resection of CRC. These findings strongly suggest that CD26 may be a useful prognostic marker in patients with CRC. Other investigators demonstrated in a murine model that lung metastasis of CRC was suppressed by treatment with a DPPIV inhibitor (Jang et al., 2015). They showed a reduction of EMT markers, suggesting that the EMT status of the murine colon cancer cell line MC38 was at least in part affected by DPPIV inhibition, with a diminution in the growth of metastases. They also showed that DPPIV inhibition decreased the growth of lung metastases of colon cancer by downregulating autophagy, increasing apoptosis and arresting the cell cycle.

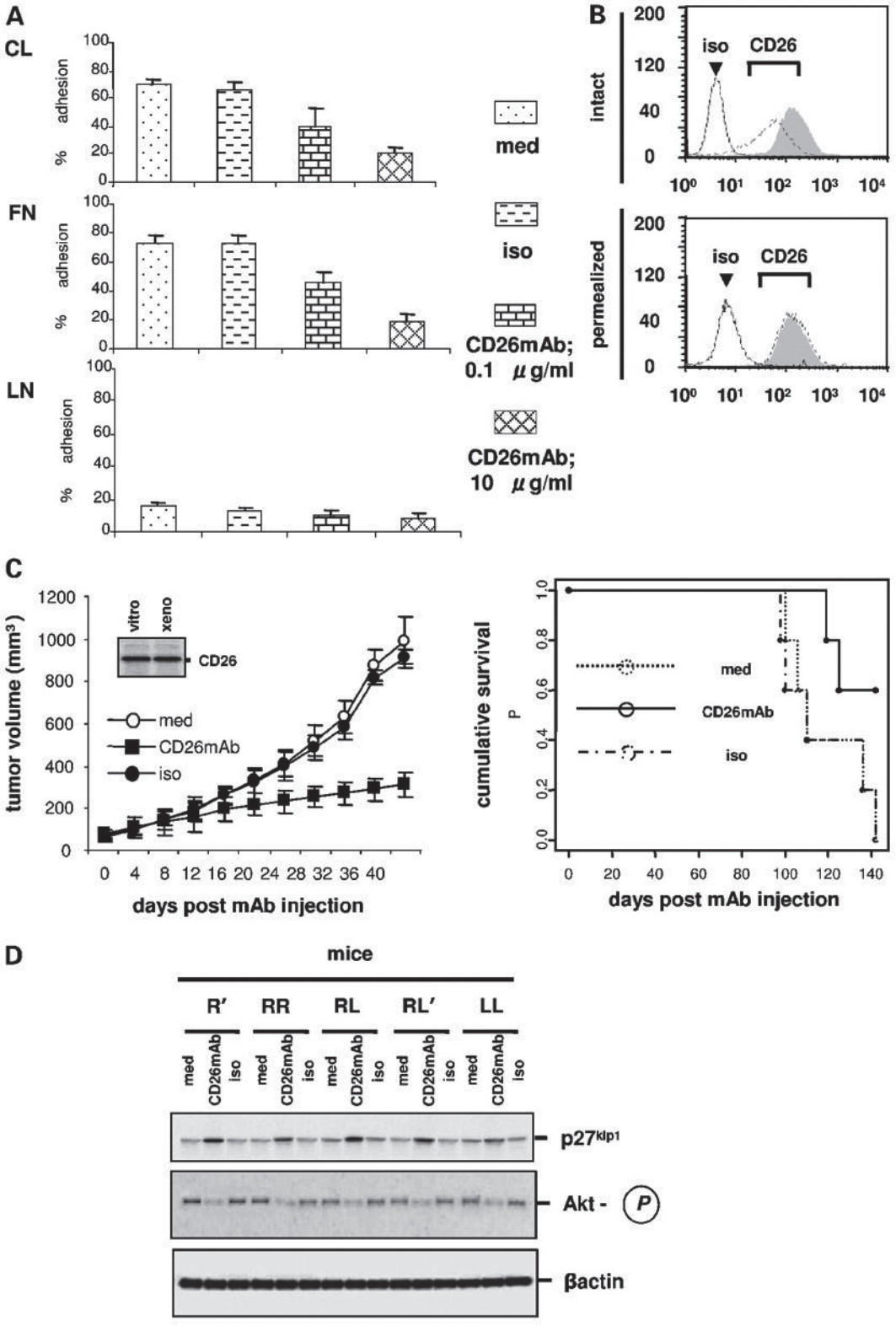

Figure 1. Antitumor effect of anti-CD26 mAb in mouse xenograft model of Caki-2, human renal carcinoma. (A) effect of anti-CD26 murine mAb (14D10) on cell adhesion to ECM. Caki-2 cells treated with medium only, anti-CD26 mAb, or isotype-matched control mAb were plated onto 60-mm dishes (3 x 10^6 per dish) coated with collagen I (*CL*), fibronectin (*FN*), or laminin (*LN*) and cultured for 21 hours. The

adhesive ability of cancer cells was expressed as the mean number of cells that had attached to the bottom surface of the dish. Columns, mean number of cells per field of view; bars, SE. Values for invasion were determined by calculating the average number of adhesive cells per mm^2 over three fields per assay and expressed as an average of triplicate determinations. Adhesive cells (%): adhesive cells / adhesive cells + nonadhesive cells. (B) Caki-2 cells were treated with anti-CD26 mAb on ice, or isotype-matched control mAb, followed by washing in ice-cold PBS twice and subsequently incubated at 37°C for 12 hours. Cells were collected and stained with FITC-conjugated anti-mouse IgG. Expression status of cell surface CD26 was analyzed by flow cytometry. To detect total CD26 level, including the internalized CD26 fraction, cell membrane permeabilization method was used. Filled histogram, positive control, which was incubated 30 minutes with anti-CD26 mAb. Open histogram, status of CD26 after treatment. (C) Caki-2 cells (1×10^6) were inoculated s.c. into the left flank of mice. CD26 expression of Caki-2 cells after tumor implantation into the mouse was similar to its level before tumor implantation. Mice were treated with intratumoral injection of PBS only (medium; $n = 5$), anti-CD26 mAb ($n = 5$), or isotype-matched control mAb ($n = 5$) on the day when the tumor mass became visible (5 mm in size). Tumor size and cumulative survival were monitored. (D) resected specimens were immediately frozen for whole-cell lysate preparation and lysed by lysis buffer. Protein (50 μg) was applied for SDS-PAGE and immunoblotting for $p27^{kip1}$, phosphorylated Akt, and β-actin. R,' RR, RL, RL,' and LL, names of mice in each treatment group. This figure is reprinted with permission from Inamoto T et al., *Clin Cancer Res* 12: 3470-7, 2006.

2.2.4. T-Anaplastic Large Cell Lymphoma and Acute T Cell Leukemia

We showed that treatment with anti-CD26 murine mAb inhibited adhesion of the human $CD30^+$ T-anaplastic large cell lymphoma cell line Karpas 299 to fibronectin. Furthermore, depletion of CD26 in Karpas 299 cells by siRNA decreased tumorigenesis and increased survival of SCID mice inoculated with these cells (Sato et al., 2005). In addition, we demonstrated that anti-CD26 murine mAb treatment of the CD26-positive Karpas 299 resulted in *in vitro* and *in vivo* anti-tumor activity. Administration of anti-CD26 mAb induced cell cycle arrest at the G1/S checkpoint, associated with enhanced p21 expression, and significantly enhanced survival of SCID mice inoculated with Karpas 299 cells by inhibiting tumor formation (Ho et al., 2001). Likewise, we showed that treatment with anti-CD26 murine mAb inhibited the growth of acute T cell leukemia cell line Jurkat transfected with CD26 through G1/S cell cycle arrest, associated with concurrent activation of the ERK signaling pathway and increased p21 expression (Ohnuma et al., 2002). These findings further

support a therapeutic approach involving targeted therapy against CD26 for selected hematological malignancies.

2.2.5. Gastrointestinal Stromal Tumor

Yamaguchi et al., comprehensively investigated gene expression profiles in surgical samples of untreated GIST of the stomach and small intestine. They found that the disease-free survival of patients with CD26-positive GIST of the stomach was worse than that of patients with CD26-negative GIST (Yamaguchi et al., 2008). Moreover, the postoperative recurrence rate of CD26-negative gastric GIST cases was as low as 2.0%. Meanwhile, CD26 expression was not associated with clinical outcome of small intestinal GIST. They concluded that CD26 is a significant prognostic factor of gastric GIST and may also serve as a therapeutic target (Yamaguchi et al., 2008).

2.2.6. Thyroid Carcinoma

The *BRAF*V600E mutation, which results in greater mitogen-activated protein kinase signaling output, is the most predominant oncogenic driver of thyroid cancer (Fagin and Wells, 2016). CD26 expression is upregulated in malignant thyroid tumors, and CD26 can be used as a malignancy marker in fine-needle aspiration cytology of thyroid nodules (de Micco et al., 2008). Recently, CD26, secretogranin V (SCG5) and carbonic anhydrase XII (CA12) are a three-gene signature that can distinguish malignant thyroid cancers, and useful for preoperative diagnosis of thyroid cancer (Zheng et al., 2015). More recently, the function of CD26 in thyroid cancer has been investigated. High CD26 was associated with extrathyroidal extension, *BRAF* mutation, and advanced tumor stage in papillary thyroid cancer (Lee et al., 2017). CD26 silencing by siRNA or treatment with DPPIV inhibitors significantly suppressed colony formation, cell migration, and invasion of thyroid cancer. CD26 expression was suggested to be involved in the transforming growth factor (TGF)-β signaling pathway. Furthermore, *in vivo* experiments revealed that treatment with the DPPIV inhibitor sitagliptin reduced tumor growth and xenograft TGF-β receptor I expression (Lee et al., 2017). These

investigators concluded that increased CD26 expression is associated with cellular invasion and more aggressive disease in papillary thyroid cancer, and targeting CD26/DPPIV may be a therapeutic strategy for CD26-expressing thyroid cancer.

2.2.7. Urothelial Carcinoma

Although there is an increasing number of biomarkers that have prognostic relevance to urothelial carcinoma (UC), factors involved in tumor progression remained largely unclear. Recently, by mining the datasets obtained from the Gene Expression Omnibus (GEO, NCBI, Bethesda) and focusing on the proteolysis pathway, Liang et al., discovered that mRNA level of CD26 is significantly upregulated in advanced-stage human UC and the upregulation of CD26 is most significantly associated with clinical aggressiveness of UC (Liang et al., 2017). CD26 is an independent prognostic biomarker for disease-specific survival and metastasis-free survival. Moreover, CD26 knockdown by shRNA resulted in a significantly decreased cell viability, proliferation, migration, and invasion in urothelial cell lines, J82 and RTCC-1 cells (Liang et al., 2017). These findings strongly suggest that CD26 plays a role in the aggressiveness of UCs, and can serve as a novel prognostic marker and therapeutic target.

2.2.8. Chronic Myeloid Leukemia

Chronic myeloid leukemia (CML) is a stem cell neoplasm characterized by the BCR/ABL1 oncogene. Herrmann et al., recently identified CD26/DPPIV as a novel, specific and pathogenetically relevant biomarker of $CD34^{+}CD38^{-}$ CML leukemic stem cell (LSC) (Herrmann et al., 2014). CD26 was not detected on normal stem cells or LSC in other hematopoietic malignancies. Correspondingly, $CD26^{+}$ LSC decreased to low or undetectable levels during successful treatment with imatinib. $CD26^{+}$ CML LSC engrafted NOD-SCID-IL-2R$\gamma^{-/-}$ (NSG) mice with $BCR/ABL1^{+}$ cells, whereas $CD26^{-}$ LSC from the same patients produced multilineage $BCR/ABL1^{-}$ engraftment. Moreover, targeting of CD26 by gliptins suppressed the expansion of $BCR/ABL1^{+}$ cells. These results

suggest that CD26 is a new biomarker and target of CML LSC, and inhibition of CD26/DPPIV may revert abnormal LSC function and support curative treatment approaches in this malignancy. More recently, Warfvinge et al., extensively defined the heterogeneity within the LSC population in chronic phase CML patients at diagnosis and following conventional tyrosine kinase inhibitor (TKI) treatment. Cell surface expression of the CML stem cell markers CD25, CD26, and IL1RAP was high in all subpopulations at diagnosis but downregulated and unevenly distributed across subpopulations in response to TKI treatment. The most TKI-insensitive cells of the LSC compartment could be captured within the $CD45RA^-$ fraction and further defined as positive for CD26 in combination with an aberrant lack of cKIT expression. These results expose a considerable heterogeneity of the CML stem cell population and propose a $Lin^-CD34^+CD38^{-/low}CD45RA^-cKIT^-CD26^+$ population as a potential therapeutic target for improved therapy response (Warfvinge et al., 2017).

2.3. Mechanisms of Action of YS110 for Cancer Treatment

We had developed a novel humanized anti-CD26 mAb, namely YS110. YS110 is a recombinant DNA-derived humanized mAb that selectively binds with high affinity to the extracellular domain of CD26. The antibody is an $IgG_1\kappa$ with a molecular weight of 144 kDa and was humanized via an *in silico* design based on the amino acid sequence of anti-human CD26 murine mAb (14D10), which inhibited tumor cell growth, migration and invasion, and enhanced survival of mouse xenograft models (Inamoto et al., 2006). YS110 is produced by fermentation in CHO (Chinese hamster ovary) mammalian cell suspension culture with the Glutamine Synthetase Expression System. *In vitro* pharmacologic evaluation of YS110 demonstrated its selective binding to human CD26 on a number of human cancer cell lines and tissues, with no apparent agonistic effect on human CD26-positive lymphocytes and no inhibition of DPPIV activity. Moreover, in the proof-of-concept (POC) studies using preclinical models, *in vivo* administration of YS110 resulted in inhibition of tumor cell growth,

migration and invasion, and enhanced survival of mouse xenograft models inoculated with RCC or MPM (Inamoto et al., 2006; Inamoto et al., 2007; Okamoto et al., 2014; Yamamoto et al., 2014). In addition to our robust *in vitro* and *in vivo* data on antibody-mediated dose-dependent tumor growth inhibition, YS110 exhibited excellent safety and pharmacological profiles in non-human primate models using single and repeated increasing intravenous doses. Other key safety findings were obtained from studies involving cynomolgus monkeys which express YS110-reactive CD26 molecules, with similar tissue distribution profiles and expression levels to human CD26. We therefore conducted the FIH phase 1 clinical trial of YS110 for patients with refractory MPM and other CD26-positive solid tumors (Angevin et al., 2017). Thirty-three heavily pre-treated patients with CD26-positive cancers including 22 MPM, 10 RCC and 1 UC underwent YS110 administration. Our FIH phase 1 study conclusively demonstrated that YS110 exhibits a favorable safety profile and substantial clinical activity in heavily pre-treated CD26-positive MPM and RCC patients who had previously progressed on conventional standard chemotherapies. Further clinical trial of YS110 for MPM is in progress worldwide (ClinicalTrials.gov, 2017).

The role of CD26 in cancer cell biology and the mechanisms of action of YS110 for cancer treatment have been reviewed in detail recently (Ohnuma et al., 2017). In brief, we summarize the mechanisms of action of YS110 as follows; (i) a direct cytotoxic effect on certain human CD26-positive cancer cell lines via antibody-dependent cell-mediated cytotoxicity (ADCC) (Inamoto et al., 2007), (ii) a direct anti-tumor effect through the induction of cell cycle arrest by induction of $p27^{kip1}$ and $p21^{cip1}$ expression (Inamoto et al., 2007; Hayashi et al., 2016), (iii) the nuclear translocation of CD26 molecules by internalization of the CD26-YS110 complexes to inhibit proliferation of tumor cells via suppression of *POLR2A* gene expression, a component of RNA polymerase II (Yamada et al., 2009; Yamada et al., 2013), and (iv) following internalization of the CD26-YS110 complexes, an inhibition of invasion and migration of tumor cells by decreased binding to the collagen/fibronectin microenvironment matrix (Inamoto et al., 2007). We are currently investigating other

mechanisms of action for the observed anti-tumor activity of YS110, especially focusing on the effects of YS110 on tumor immunology.

The FIH phase 1 clinical study of YS110 revealed that an increase in YS110 infusion dose was associated with decreased serum sCD26 level and DPPIV enzyme activity, particularly in cohorts 4-6 (2.0 to 6.0 mg/kg), with an approximately 80% decrease in these levels (Angevin et al., 2017). Although DPPIV inhibitors are clinically used as oral hypoglycemic agents (Drucker and Nauck, 2006), hypoglycemia was not observed during YS110 administration. Of note is the fact that greater than 80% inhibition of serum DPPIV activity was obtained 24 hours after oral administration of clinically available DPPIV inhibitors (drug information published by manufacturers of sitagliptin, vildagliptin, saxagliptin and *etc*.), a level of inhibition comparable to that seen in patients treated with YS110. Our current data would therefore indicate that YS110 therapy is tolerable in the clinical setting. Recent work has demonstrated the functional role of DPPIV-mediated post-translational modification of chemokines in regulating tumor immunity through its interaction with its substrate CXCL10 (Barreira da Silva et al., 2015). Barreira da Silvia et al., used *in vivo* tumor-transplant models to show that DPPIV inhibition reduced tumor growth through the preservation of bioactive CXCL10 in the tumor microenvironment (TME). In the normal physiological state, CXCL10 is rapidly degraded by DPPIV, resulting in decreased recruitment and migration of $CXCR3^+$ T cells into the TME. CXCR3 has been shown to be a functional receptor for CXCL10 (Proost et al., 2001). In contrast, DPPIV inhibition enhanced tumor rejection by preserving the full-length biologically active form of CXCL10, leading to increased trafficking of $CXCR3^+$ T cells into the TME (Ohnuma et al., 2015b). This anti-tumor response is potentiated in combination with other anti-tumor immunotherapeutic approaches including CpG adjuvant therapy, adoptive T cell transfer therapy and checkpoint blockade therapy (anti-CTLA-4 and anti-PD-1) (Barreira da Silva et al., 2015). In view of these recent findings, data from our current trial showing that serum DPPIV activity was decreased following treatment with YS110 in a dose-dependent manner would suggest that anti-tumor activity via DPPIV inhibition may constitute

yet another mechanism of action for the anti-tumor activity of YS110 (Ohnuma et al., 2015b).

Although the cellular and molecular mechanisms involved in CD26-mediated T cell activation have been extensively evaluated by our group and others (Morimoto and Schlossman, 1998; Ohnuma et al., 2008a; De Meester et al., 1999), potential negative feedback mechanisms to regulate CD26-mediated activation still remain to be elucidated. Utilizing human peripheral blood lymphocytes, we recently found that CD26-mediated costimulation induced the development of a population of human type 1 regulatory T (T_{reg}) cells from $CD4^+$ T cells with high level of IL-10 production and lymphocyte-activation gene 3 (LAG3) expression (Hatano et al., 2015). Other investigators have also reported that the $CD26^+CD39^-$ T_{reg} subset among $CD4^+$ T_{reg} exhibits high level of IL-10 expression (Hua et al., 2015). These findings strongly suggest that a specific subset of $CD26^+$ T cells plays a role in immune checkpoint system, and that the CD26 molecule may be a novel target for a therapeutic approach involving immune checkpoint blockade. We are currently investigating whether CD26-mediated signals are associated with the induction of immune checkpoint molecules in the tumor-infiltrating lymphocytes, and definitive data will be presented in the near future.

3. IMMUNE DISORDERS

3.1. Acute Graft-versus-Host Disease

GVHD is a severe complication and major cause of morbidity and mortality following allogeneic hematopoietic stem cell transplantation (alloHSCT) (Giralt, 2012). GVHD results from an immunobiological attack on target recipient organs by donor allogeneic T cells that are transferred along with the allograft (Blazar et al., 2012; Shlomchik, 2007). The pivotal role of donor-derived T cells in acute GVHD (aGVHD) is supported by the complete abrogation of GVHD following T cell depletion from the graft (Giralt, 2012; Tsirigotis et al., 2012). This approach remains

the most effective in preventing aGVHD. However, complications such as graft rejection, loss of GVL effect or increased opportunistic infections can occur. Although new approaches are being developed in the clinical setting for the prevention or treatment of GVHD (Blazar et al., 2012), in-depth understanding of the precise cellular mechanisms of human GVHD is necessary for more effective and less immunocompromising strategies to improve the clinical outcome of alloHSCT.

Costimulatory pathways are required to induce T cell proliferation, cytokine secretion and effector function following antigen-mediated T cell receptor activation (Rudd, 2010), and the important role of costimulatory pathways in transplant biology has been established (Markey et al., 2014). The most extensively studied pathways involve interactions between CD28 and the B7 molecules CD80 and CD86 (Rudd et al., 2009). Initial studies focused on the *in vivo* blockade of interactions between CD28 or cytotoxic T lymphocyte antigen 4 (CTLA4) and their B7 ligands, CD80 and CD86, using a CTLA4-immunoglobulin fusion protein (CTLA4-Ig) or B7-specific antibodies (Lenschow et al., 1992; Saito, 1998). Blockade of CD28 pathways may lead to profound immunosuppression (Blazar et al., 1997). CD26 is also associated with T cell signal transduction processes as a costimulatory molecule, as well as being a marker of T cell activation (Morimoto and Schlossman, 1998; Ohnuma et al., 2008a). In fact, patients with autoimmune diseases such as multiple sclerosis, Grave's disease, and RA have been found to have increased numbers of $CD4^+CD26^+$ T cells in inflamed tissues as well as in their peripheral blood (Eguchi et al., 1989; Gerli et al., 1996; Hafler et al., 1985; Mizokami et al., 1996), with enhancement of CD26 expression in these autoimmune diseases correlating with disease severity (Eguchi et al., 1989; Gerli et al., 1996; Muscat et al., 1994). Moreover, $CD26^{high}CD8^+$ T cells in humans belong to early effector memory T cells, and $CD26^{high}CD8^+$ T cells exhibited increased expression of granzyme B, TNF-α, IFN-γ and Fas ligand, and exerted cytotoxic effect with CD26-mediated costimulation (Hatano et al., 2013b). These findings implied that $CD26^+$ T cells play an important role in the inflammation process and subsequent tissue damage in such diseases, and suggested that

$CD26^+$ T cells belong to the effector T cell population. However, little is known about the effectiveness of CD26-targeting therapy on aGVHD.

To determine whether human $CD26^+$ T cells play a role in an animal model of inflammatory diseases mediated by human effector lymphocytes, we utilized a xenogeneic model of GVHD resulting from the adoptive transfer of human peripheral blood mononuclear cells (PBMCs) into NOD/Shi-*scid*, IL-2Rγ^{null} (NOG) mice (hu-PBL-NOG mice) (Ito et al., 2002). We first conducted a pathological analysis of x-GVHD target organs such as the skin, colon or liver in hu-PBL-NOG mice. The liver, colon and skin of hu-PBL-NOG mice were infiltrated with human $CD3^+$ mononuclear cells (MNCs), with associated organ destruction. Moreover, human $CD3^+$ MNCs reactive to anti-human CD26 Ab were readily visible in all evaluated samples in the liver, colon or skin of x-GVHD mice. The infiltrated $CD26^+$ cells were confirmed to be human $CD3^+$ T cells by co-staining analysis with flow cytometry. These results suggest that donor-derived human $CD26^+$ cells play a role in the pathogenesis of x-GVHD in our hu-PBL-NOG murine model.

We attempted to determine whether treatment with humanized anti-CD26 mAb could ameliorate disease progression and severity in our x-GVHD murine model. As comparison, the clinically available T cell costimulation blocking agent CTLA4-Ig (abatacept) was utilized (Genovese et al., 2005; Gribben et al., 1996). As shown in Figure 2A, anti-CD26 mAb (orange or red lines) or CTLA4-Ig (green or dark green lines) treatment of hu-PBL-NOG mice significantly increased overall survival, as compared with control IgG treatment (blue line). This life-prolonging effect of anti-CD26 mAb was observed at the same low dose as CTLA4-Ig (orange or green lines in Figure 2A, respectively). Moreover, GVHD-associated weight loss for up to 4 weeks post cell inoculation was not significantly different between anti-CD26 mAb and CTLA4-Ig treatment groups (orange or green lines in Figure 2B). These data strongly suggest that anti-CD26 mAb treatment is a promising novel therapeutic agent for x-GVHD, with efficacy comparable to CTLA4-Ig.

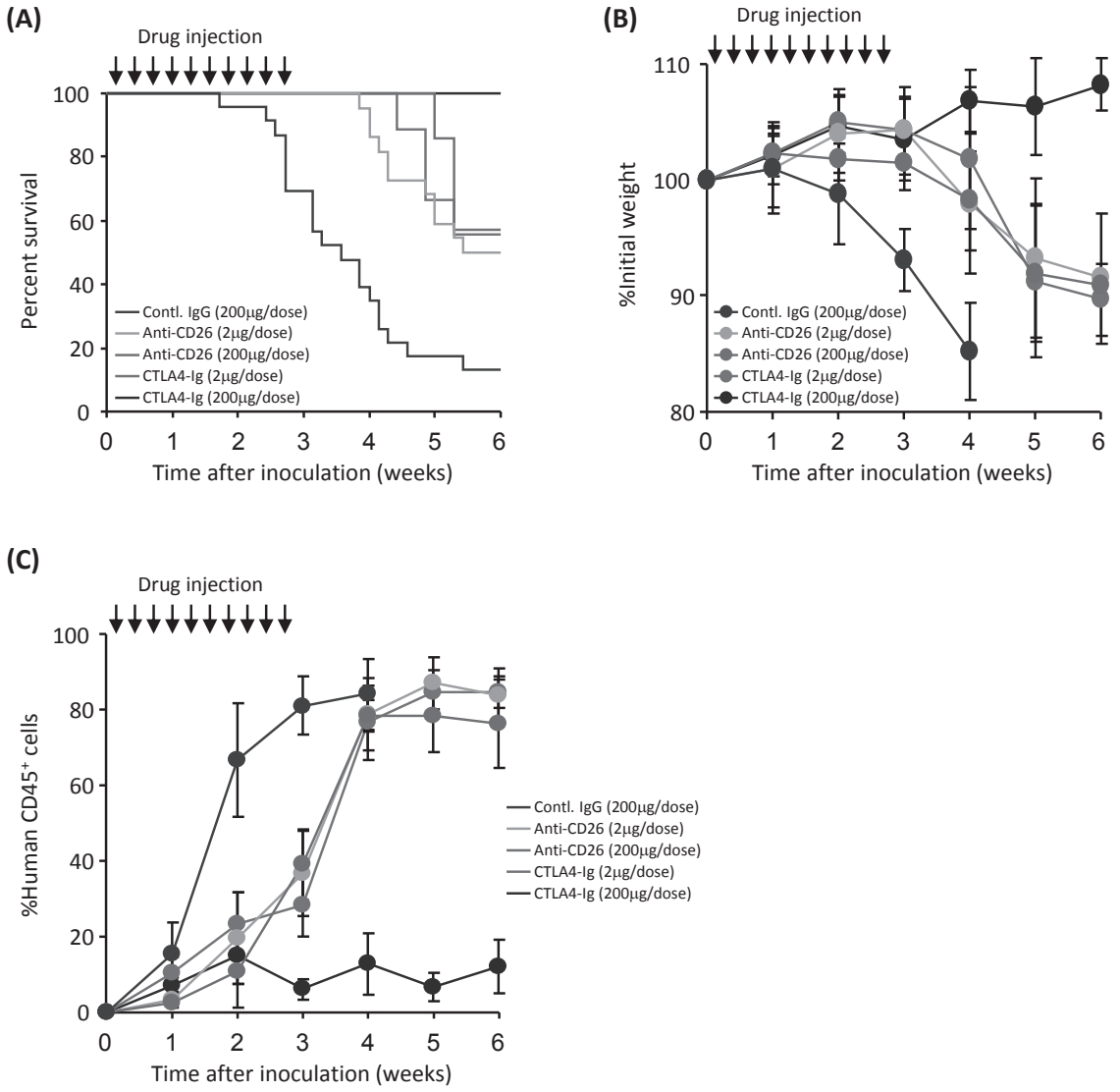

Figure 2. Anti-CD26 mAb treatment reduces x-GVHD-related lethality, and weight loss in hu-PBL-NOG mice without rejection of donor-derived human lymphocytes. After 1 day of inoculation of human PBMCs, Hu-PBL-NOG mice were injected intraperitoneally with humanized anti-CD26 mAb, CTLA4-Ig, or isotype IgG control at indicated doses in 200 μl of sterile phosphate-buffered saline (PBS), and then were injected thrice weekly for a total of 10 doses to assess potency in preventing x-GVHD. (A) Kaplan–Meier survival curves for mice receiving PBMC plus control IgG (200 μg/dose, blue line, n = 23), low dose anti-CD26 mAb (2 μg/dose, orange line, n = 24) (P = 0.0001 vs. control IgG group), high dose anti-CD26 mAb (200 μg/dose, red line, n = 7) (P = 0.0006 vs. control IgG group), low dose CTLA4-Ig (2 μg/dose, green line, n = 9) (P = 0.0005 vs. control IgG group), or high dose CTLA4-Ig (200 μg/dose, dark green line, n = 6) (P = 0.0008 vs. control IgG group). (B) Average weight (percentage ± standard deviation (SD) of initial) for mice surviving on a given day for different groups of mice as shown in (A). (C) Time course changes of average percentage (± SD) of human $CD45^+$ lymphocytes in peripheral blood in mice receiving PBMC

plus control IgG (200 μg/dose, blue line, n = 23), low dose anti-CD26 mAb (2 μg/dose, orange line, n = 24), high dose anti-CD26 mAb (200 μg/dose, red line, n = 7), low dose CTLA4-Ig (2 μg/dose, green line, n = 9), or high dose CTLA4-Ig (200 μg/dose, dark green line, n = 6). This figure is reprinted with permission from Hatano R et al., *Br J Haematol* 162: 263-77, 2013.

We next analyzed circulating human lymphocytes in the peripheral blood of hu-PBL-NOG mice receiving anti-CD26 mAb or CTLA4-Ig. As shown in Figure 2C, at 3 weeks after inoculation, human lymphocyte level was increased in peripheral blood of mice receiving anti-CD26 mAb at both low dose and high dose (orange and red lines of Figure 2C), comparable to low dose CTLA4-Ig (green line of Figure 2C). On the other hand, in mice receiving high dose CTLA4-Ig, most of the inoculated human PBMCs were rejected (dark green line in Figure 2C). These data indicate that while treatment with increasing doses of CTLA4-Ig resulted in the absence of x-GVHD development, graft rejection did occur, potentially analogous to findings in a clinical trial with a new CTLA4-Ig agent, belatacept (Vincenti et al., 2010). On the other hand, increasing doses of anti-CD26 mAb resulted in no graft rejection in hu-PBL-NOG mice with prolonging survival as compared to hu-PBL-NOG mice receiving control IgG.

We next examined human CD26 expression level in the peripheral blood of hu-PBL-NOG mice receiving anti-CD26 mAb or CTLA4-Ig. Higher expression level of human CD26 on both $CD4^+$ and $CD8^+$ human lymphocytes in the peripheral blood of hu-PBL-NOG mice receiving control IgG or CTLA4-Ig was observed from 1 to 3 weeks after inoculation. On the other hand, in hu-PBL-NOG mice receiving anti-CD26 mAb, neither $CD4^+$ nor $CD8^+$ T cells expressed CD26 from 1 to 3 weeks. At 3 weeks after inoculation, very weak expression of human CD26 was observed on $CD4^+$ or $CD8^+$ cells in the peripheral blood of hu-PBL-NOG mice receiving anti-CD26 mAb. Taken together, these data suggest that decreased number of $CD26^{high}$ effector T cells may be responsible for the relative absence of x-GVHD development in mice receiving anti-CD26 mAb.

Since $CD26^{high}$ effector lymphocytes have high capacity for migration into inflamed tissues, we examined liver tissues of hu-PBL-NOG mice for

donor lymphocyte infiltration. In x-GVHD mice receiving control IgG, liver damage was observed with infiltration of human $CD4^+$ or $CD8^+$ MNCs. Moreover, slight inflammation of the portal duct areas in the liver was observed in mice receiving CTLA4-Ig. On the other hand, infiltration of human T cells in the liver was barely detected in mice receiving anti-CD26 mAb. In addition to the pathological changes seen in the liver, significant elevation of serum alanine aminotransferase (ALT) activity was observed in mice receiving control IgG or CTLA4-Ig, while that of mice receiving anti-CD26 mAb was found to be near normal level. Along with lymphocyte infiltration in the GVHD target tissues, the mRNA level of effector cytokines of donor-derived human $CD4^+$ or $CD8^+$ cells in the liver of hu-PBL-NOG mice receiving anti-CD26 mAb was decreased compared to those of hu-PBL-NOG mice receiving control IgG. Taken together, these results indicate that anti-CD26 treatment in hu-PBL-NOG mice ameliorated liver GVHD by decreasing production of proinflammatory cytokines of donor-derived human lymphocytes as well as inhibiting lymphocyte infiltration in the liver.

Since aGVHD and GVL effects are immune reactions highly linked to each other (Wu and Ritz, 2009; Zorn et al., 2002), we evaluated the potential influence of anti-CD26 mAb treatment on GVL effect. NOG mice transplanted with P815 cells along with human PBMCs and control IgG showed minimal signs of tumor growth in the inoculated region, but all mice died around 4 weeks after inoculation due to x-GVHD. On the other hand, mice inoculated with P815 along with human PBMC and anti-CD26 mAb exhibited enhanced survival rate with minimal evidence of GVHD. Importantly, mice in this group showed significantly slow initial tumor growth, suggesting the preservation of GVL effect more than CTLA4-Ig treatment. We examined the expression level of effector cytokines of human $CD8^+$ T cells isolated from the spleens of hu-PBL-NOG mice at 2 weeks after transplantation. Our data suggest that the GVL effect of cytotoxic effector function occurring at the early time period prior to manifestation of x-GVHD was preserved in hu-PBL-NOG mice receiving anti-CD26 mAb, compared to those in hu-PBL-NOG mice receiving CTLA4-Ig. In conclusions, CD26-mediated T cell activation

appears to play a significant role in GVHD. Since full suppression of x-GVHD with interventional therapies is currently a difficult challenge, our data demonstrating that control of x-GVHD can be achieved by modulating $CD26^{high}$ T cells with anti-CD26 mAb are potentially important clinically. Our work also suggests that anti-CD26 mAb treatment may be a novel therapeutic approach for GVHD in the future.

3.2. Chronic Graft-versus-Host Disease

On the basis of differences in clinical manifestations and histopathology, GVHD can be divided into acute and chronic forms (Blazar et al., 2012). aGVHD and chronic GVHD (cGVHD) are traditionally diagnosed primarily by time of onset, with cGVHD occurring after day 100 of transplantation (Deeg et al., 1997). However, cGVHD has distinct clinicopathologic features and is often diagnosed based on these features regardless of time of onset, being characterized by cutaneous fibrosis, involvement of exocrine glands, hepatic disease, and obliterative bronchiolitis (OB) (Filipovich, 2008; Socie and Ritz, 2014). OB, characterized by airway blockade, peribronchiolar and perivascular lympho-fibroproliferation and obliteration of bronchioles, is a late-stage complication of cGVHD (Chien et al., 2010). Patients diagnosed with OB have a 5-year survival rate of only 10 to 40%, compared to more than 80% of patients without OB (Dudek et al., 2003; Nakaseko et al., 2011). Furthermore, while multiple strategies to control cGVHD involving T cell depletion from the graft or global immunosuppression have been developed, cGVHD is still a common clinical outcome in many alloHSCT patients (Socie and Ritz, 2014; Zeiser and Blazar, 2016). In addition, immunosuppression potentially abrogates the GVL effect, associated with increased relapses following alloHSCT (Champlin et al., 1999). Novel therapeutic approaches are thus needed to control cGVHD without eliminating the GVL effect.

Since our aGVHD model described in the preceding section succumbed to aGVHD around 4 weeks after transplantation of human adult

PBL, this early-onset model of aGVHD does not permit the assessment of longer term consequences of interventional therapies, such as their effect on the development of OB, a form of cGVHD of the lung. In contrast to adult PBL, human cord blood (HuCB) lymphocytes have been reported to be immature, predominantly consisting of $CD45RA^+$ naive cells (Kobayashi et al., 2004). We previously showed that, while all HuCB CD4 T cells constitutively express CD26, CD26-mediated costimulation was considerably attenuated in HuCB CD4 T cells, compared to the robust activation via CD26 costimulation of adult PBL (Kobayashi et al., 2004). Based on these findings, we hypothesized that HuCB naive CD4 T cells gradually acquire a xenogeneic response via attenuated stimulatory signaling with indolent inflammation in the target organs, leading eventually to chronic inflammatory changes. We therefore succeeded in developing a humanized murine pulmonary cGVHD model utilizing NOG mice as recipients and HuCB as donor cells (Ohnuma et al., 2015a), and overcame the limitations seen in the humanized murine aGVHD model such as vigorous activation of all engrafted T cells and extensive loss of B cell maturation and activation (Shultz et al., 2012). Whole CB transplant mice exhibited clinical signs/symptoms of GVHD as early as 4 weeks post transplant, and demonstrated significantly decreased survival rate. The lung of whole CB transplant mice showed perivascular and subepithelial inflammation and fibrotic narrowing of the bronchiole. Skin of whole CB transplant mice manifested fat loss, follicular drop-out and sclerosis of the reticular dermis in the presence of apoptosis of the basilar keratinocytes while the liver exhibited portal fibrosis and cholestasis. These findings indicate that whole CB transplant mice develop pulmonary cGVDH as well as concomitant active GVHD in skin and liver. Taken together, our data demonstrate that the lung of whole CB transplant mice exhibits OB as manifestation of pulmonary GVHD (Ohnuma et al., 2015a).

Utilizing this model, we identified IL-26 as a key effector cytokine inducing transplant-related obliterative bronchiolitis. Lung of HuCB mice exhibited obliterative bronchiolitis with increased collagen deposition and predominant infiltration with human $IL\text{-}26^+CD26^+$CD4 T cells. Moreover, although IL-26 is absent from rodents, we showed that IL-26 increased

collagen synthesis in fibroblasts and promoted lung fibrosis in a murine GVHD model using *IL-26* transgenic mice. *In vitro* analysis demonstrated a significant increase in IL-26 production by HuCB CD4 T cells following CD26 costimulation, whereas Ig Fc domain fused with the N-terminal of caveolin-1 (Cav-Ig), the ligand for CD26, effectively inhibited production of IL-26. Administration of Cav-Ig before or after onset of GVHD impeded the development of clinical and histologic features of GVHD without interrupting engraftment of donor-derived human cells, with preservation of the GVL effect (Ohnuma et al., 2015a). These results therefore provide proof of principle that cGVHD of the lungs is caused in part by IL-$26^{+}$$CD26^{+}$CD4 T cells, and that blockade of CD26-caveolin-1 interaction by Cav-Ig or YS110 could be beneficial for cGVHD prevention and therapy.

3.3. Rheumatoid Arthritis

RA is a chronic, inflammatory autoimmune disease that primarily affects the joints, but also has systemic symptoms. RA is characterized by progressive invasion of synovial fibroblasts into the articular cartilage and erosion of the underlying bone, followed by joint destruction (Asif Amin et al., 2017). Several reports on RA patients have shown that concentration of soluble CD26 and DPPIV enzyme activity were significantly decreased in both synovial fluid and serum compared with osteoarthritis patients or healthy donors (Busso et al., 2005; Buljevic et al., 2013; Cordero et al., 2015). Cordera et al., studied serum levels of IL-12, IL-15, and soluble CD26 from 35 patients with active and inactive RA as well as those of healthy controls (Cordero et al., 2001). Patients' sera had higher IL-12 and IL-15 levels, and the level of soluble CD26 was inversely correlated with the number of swollen joints. These findings suggest that these cytokines and CD26 are associated with the inflammation and immune activity in RA. A number of proinflammatory peptides are supposed to be involved in the pathogenesis of RA, and have their biological activity controlled by limited proteolysis mediated by DPPIV and DPPIV-like hydrolytic activity (Wolf

et al., 2008). Several investigators have demonstrated that SDF-1α (CXCL12), a substrate of DPPIV, is a crucial mediator controlling the influx of lymphocytes and monocytes/macrophages into the inflamed synovium of RA and thus triggering joint destruction (Kim et al., 2007; Sromova et al., 2010). DPPIV regulates neuropeptide Y (NPY) and vasoactive intestinal peptide (VIP), recently implicated in RA (Buljevic et al., 2013). However, involvement of DPPIV enzyme activity in the pathology of RA remains controversial. Using CD26/DPPIV-KO mice, Busso et al., demonstrated that damage severity, as indicated by synovial thickness, knee-joint inflammation, and histological grading, was more severe in CD26/DPPIV-KO mice than in wild-type control mice in antigen- and collagen-induced arthritis models (Busso et al., 2005). The authors concluded that decreased circulating CD26/DPPIV levels in arthritis increased the intact active form of SDF-1, associated with increased numbers of CXCR4 (SDF-1 receptor)-positive cells infiltrating arthritic joints. On the other hand, using a type II collagen-induced or alkyldiamine-induced rat model of arthritis, Tanaka et al., showed that treatment with any DPPIV inhibitors examined reduced hind paw swelling, an indicator of disease severity (Tanaka et al., 1997; Tanaka et al., 1998).

In contrast with the decrease in the levels of soluble CD26 in the synovial fluid and serum, patients with active RA displayed higher percentage of peripheral blood $CD26^{+}CD4^{+}$ T cells than inactive RA and control subjects (Muscat et al., 1994; Cordero et al., 2015). RA synovial fluid contained lower percentages of whole $CD26^{+}$ T cells compared with peripheral blood, while the percentage of $CD26^{high}$ T cells in synovial fluid of RA was markedly increased compared with peripheral blood of RA patients and healthy subjects (Mizokami et al., 1996). These findings suggest that $CD26^{+}$ T cells induce the inflammation and tissue destruction characteristic of RA by migrating to and being active in the rheumatoid synovium. We described $CD26^{+}$ T cells infiltrating the rheumatoid synovium using immunohistochemical studies (Ohnuma et al., 2006). We also noted high expression of caveolin-1 in the rheumatoid synovium and synoviocytes. These data suggest that the CD26-caveolin-1-mediated upregulation of CD86 on activated monocytes in addition with CD26-

mediated signal transduction in T cells leads to antigen specific T cell activation in RA, and blockade of this CD26-caveolin-1 interaction by YS110 may be useful for suppressing the immune system in RA.

3.4. Inflammatory Bowel Diseases

In patients with inflammatory bowel diseases (IBD) such as Crohn's disease or ulcerative colitis, $CD26^+$ T-cells and DPPIV activity in serum were examined (Hildebrandt et al., 2001; Rose et al., 2002). In these studies, while the DPPIV activity was reduced in patients with IBD, the number of $CD25^+CD26^+$ T-cells in the peripheral blood was increased in patients with IBD. Moreover, other investigators recently reported that $CD26^{high}$ T cells contain T_H17 cells, and that $CD26^{high}$ T_H17 cells are enriched in the inflamed tissue of IBD patients (Bengsch et al., 2012). In addition, Kappeler et al., showed that activated perforin mRNA expressing T cells are present in close proximity to the intestinal epithelial cells in active stages of ulcerative colitis and Crohn's disease (Kappeler et al., 2000), suggesting an important role of cytotoxic cells in the pathogenesis of IBD. We recently showed that $CD26^{high}CD8^+$ T cells exhibit increased expression of granzyme B, TNF-α, IFN-γ and Fas ligand, and exert cytotoxic effect with CD26-mediated costimulation (Hatano et al., 2013b). Taken together, these data indicate that CD26 may be potentially important for the pathophysiology of IBD, and appears to be a useful therapeutic target for IBD.

3.5. Middle East Respiratory Syndrome Coronavirus

MERS-CoV was first identified in a 60-year-old patient in June 2012 who presented with acute pneumonia, followed by acute respiratory distress syndrome and renal failure with a fatal outcome (Zaki et al., 2012). Between 2012 and September 2017, 2080 laboratory-confirmed cases of MERS-CoV infection were reported to the World Health Organization

(WHO), which has notified of at least 722 deaths (around 35% fatality rate) related to MERS-CoV since September 2012 (WHO, 2017). Efforts to develop effective preventive and therapeutic intervention strategies are currently ongoing. Several treatment modalities have been investigated to develop effective therapies against MERS-CoV, including interferon, ribavirin, cyclosporin A, protease inhibitors, convalescent plasma and immunoglobulins. Several promising anti-MERS-CoV therapeutic agents have recently been reviewed extensively (Mo and Fisher, 2016), while broad spectrum antiviral agents might not be sufficient to treat severe MERS-CoV patients because of its limited effective therapeutic window of opportunity (Zumla et al., 2016).

An alternative approach using prophylactic regimens would be theoretically suitable to limit the spread of MERS-CoV. This scenario includes MERS vaccine and neutralizing MERS-CoV-specific mAb (Zumla et al., 2016). The MERS-CoV genome encodes for 16 non-structural proteins (nsp1-16) and 4 structural proteins, the spike (S), envelope (E), membrane (M), and nucleocapsid (N) (van Boheemen et al., 2012). The viral structural proteins, S and N, show the highest immunogenicity (Agnihothram et al., 2014). While both S and N proteins induce T-cell responses, neutralizing antibodies are almost solely directed against the S protein, with the receptor binding domain (RBD) being the major immunodominant region (Mou et al., 2013). These great challenges have been extensively reviewed previously (Zumla et al., 2016).

Recent reports indicated that the spike protein S1 of MERS-CoV is required for viral entry into human host cells (Gierer et al., 2013; Lu et al., 2013), using CD26/DPPIV as a functional receptor (Raj et al., 2013). Inhibiting virus entry into host cells could also be achieved by targeting the host receptor CD26/DPPIV. While inhibitors of the CD26/DPPIV enzymatic activity are used clinically to treat type 2 diabetes patients, commercially available DPPIV inhibitors would not serve this purpose since these agents have been shown not to inhibit binding of the RBD of MERS-CoV to CD26/DPPIV (Raj et al., 2013). We previously showed that human CD26 is a binding protein for ADA (Kameoka et al., 1993). Currently, it is known that there are two isoforms of ADA, ADA1 and

ADA2. ADA1 is particularly present in lymphocytes and macrophages, while ADA2 is found predominantly in the serum and other body fluids including pleural effusion (Gakis, 1996). CD26/DPPIV binds to ADA1, but not ADA2 (Kameoka et al., 1993). The crystal structure of CD26/DPPIV and the S protein of MERS-CoV allowed for visualization of the interacting amino acid in both proteins. However, our *in vitro* experiments showed that blockade of MERS-CoV binding to CD26/DPPIV by ADA1 is incomplete (Ohnuma et al., 2013). Therefore, mAb-mediated blocking CD26/DPPIV binding to the RBD of MERS-CoV needs to be developed.

CD26/DPPIV, a host receptor for MERS-CoV, is conserved among different species such as bats and humans, partially explaining the large host range of MERS-CoV (Mohd et al., 2016). In addition to being widely expressed in most cell types including T lymphocytes, bronchial mucosa or the brush border of proximal tubules, CD26/DPPIV exists in systemic circulation as soluble form (Ohnuma et al., 2008b). This distribution of CD26 may play a role in the systemic dissemination of MERS-CoV infection in human (Drosten et al., 2013). Therefore, an effective therapy for MERS-CoV is needed not only to block the entry of MERS-CoV into such CD26-expressing organs as the respiratory system, kidney, liver or intestine, but also to eliminate circulating MERS-CoV. In this regard, manipulation of CD26/DPPIV levels or the development of inhibitors that target the interaction between the MERS-CoV S1 domain and its receptor may provide therapeutic opportunities to combat MERS-CoV infection. More recently, the RBD in the S protein was mapped to a 231-amino acid fragment of MERS-CoV S proteins (residues 358-588) (Mou et al., 2013).

We have recently mapped MERS-CoV S protein-binding regions in human CD26 molecule and demonstrated that anti-CD26 mAbs, which had been developed in our laboratory, effectively blocked the interaction between the spike protein and CD26, thereby neutralizing MERS-CoV infectivity. To determine the specific CD26 domain involved in MERS-CoV infection, we chose six different clones of anti-CD26 mAbs (4G8, 1F7, 2F9, 16D4B, 9C11, 14D10), and the humanized anti-CD26 mAb YS110, which recognize 6 distinct epitopes of the CD26 molecule (Dong

et al., 1998; Inamoto et al., 2007), to conduct MERS-CoV S1-Fc (S1 domain of MERS-CoV fused to the Fc region of human IgG) binding inhibition assays (Ohnuma et al., 2013). 2F9 inhibited fully binding of MERS-CoV S1-Fc to JKT-hCD26WT (Jurkat cells transfected with full-length human CD26/DPPIV), while other anti-CD26 mAbs demonstrated certain levels of inhibition (1F7, or YS110) or no significant inhibition (4G8, 16D4B, 9C11 or 14D10). These results strongly suggest that the anti-CD26 mAb 2F9 has better therapeutic potential than recombinant MERS-CoV S1-Fc in preventing viral entry into susceptible cells, and that 1F7 or YS110 also blocks MERS-CoV infection.

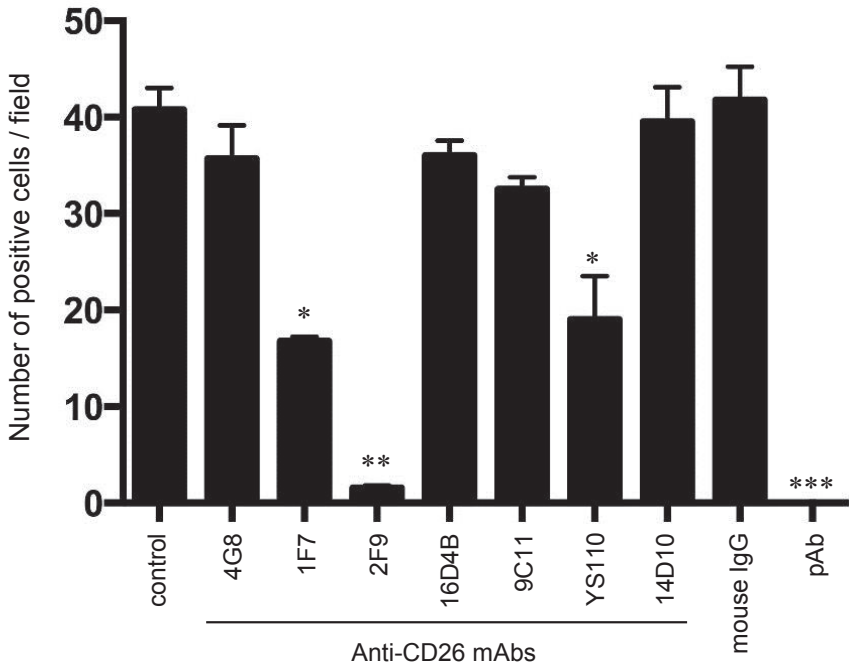

Figure 3. Inhibition of MERS-CoV infection by the anti-CD26 mAb. Huh-7 cells were preincubated with normal mouse IgG, various anti-CD26 mAbs (4G8, 1F7, 2F9, 16D4B, 9C11, or 14D10), humanized anti-CD26 mAb (YS110), or anti-CD26 goat polyclonal antibody (pAb) at a concentration of 40 μg/ml for 0.5 h prior to MERS-CoV virus inoculation (1 h), all at room temperature. Mock-incubated cells (control) were used as controls. Following incubation at 37°C for 8 h, infected cells were detected by immunofluorescence using anti-SARS-CoV NSP4 antibodies that are cross-reactive for MERS-CoV, and infection was quantified as the number of anti-SARS-CoV NSP4-positive cells. Two independent experiments were performed, and data from one representative experiment are shown. Error bars indicate SEMs (two-tailed Student's *t* test; *, **, or ***, $P < 0.05$ versus control. This figure is reprinted with permission from Ohnuma K et al., *J Virol* 87: 13892-9, 2013.

Moreover, we have characterized the CD26 epitope involved in MERS-CoV S1-Fc binding to CD26 through the use of various CD26 mutants with deletion in the C-terminal extracellular region, since CD26 is a type II transmembrane protein (Ohnuma et al., 2008a). Our biological experiments on binding regions of CD26 to MERS-CoV using mAbs showed results comparable to those obtained from crystal structure analysis (Lu et al., 2013; Wang et al., 2013). Our observations strongly suggest that the main binding regions of CD26 to MERS-CoV appear to be close to the 358th amino acid recognized by 2F9, and the regions of CD26 defined by 1F7 and YS110 (248-358th amino acids) are also partially involved in MERS-CoV binding.

To determine whether treatment with anti-CD26 mAbs 2F9 as well as 1F7 and YS110 could inhibit MERS-CoV infection, susceptible cells, Huh-7, were pre-incubated with various anti-CD26 mAbs prior to inoculation with the virus (Ohnuma et al., 2013). In this experimental system, infection was almost completely blocked by 2F9 but not by control IgG or several other anti-CD26 mAbs recognizing other epitopes (4G8, 16D4B, 14D10 or 9C11) (Figure 3). Moreover, the anti-CD26 mAbs 1F7 and YS110 considerably inhibited MERS-CoV infection of Huh-7 cells (Figure 3). These results demonstrate that 2F9 inhibits MERS-CoV entry, and therefore can potentially be developed as a preventive or therapeutic agent for MERS-CoV infection in the clinical setting. More importantly, the humanized anti-CD26 mAb YS110 has been evaluated in patients with CD26-expressing cancers in our FIH phase 1 clinical trial. Since no apparent adverse effects of YS110 have been reported besides transient and tolerable injection reactions, and moreover, the level of circulating soluble CD26 in the serum is decreased following YS110 administration (Angevin et al., 2017), YS110 may be an immediate therapeutic candidate for clinical use as potential treatment for MERS-CoV infection.

CLOSING REMARKS

CD26 is a multifunctional protein with known DPPIV enzyme activity. CD26 is expressed on various tumors including MPM. Although the exact role of CD26/DPPIV in various cancers remains to be elucidated, CD26 serves as a prognostic marker in multiple tumors such as CRC, GIST, thyroid carcinoma and UC. Moreover, in several human malignancies including MPM, CRC and CML, CD26/DPPIV expression is reported to be a marker of cancer stem cells. Our FIH phase 1 clinical trial of YS110 demonstrates that YS110 therapy exhibits a favorable safety profile and results in encouraging disease stabilization in a number of patients with advanced/refractory CD26-expressing MPM and RCC, and further clinical testing of YS110 for MPM is being conducted worldwide. Given the potential role of CD26 surface expression in cancer biology, YS110 therapy may also influence tumor growth through its potential effect on the cancer stem cells of selected tumors.

Besides being a marker of T cell activation, CD26 is also associated with T cell signal transduction processes as a costimulatory molecule and has an important role in T cell biology and overall immune function. Since CD26/DPPIV has a multitude of biological functions in human tumor cells and immune system, further detailed understanding of the role of this molecule in various clinical settings may lead potentially to novel therapeutic approaches not only for MPM but also for other refractory cancers, immune disorders and MERS-CoV infection.

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Chapter 6

CD26-TARGETED THERAPY: A NEW HORIZON IN MALIGNANT PLEURAL MESOTHELIOMA MANAGEMENT

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ABSTRACT

Malignant pleural mesothelioma (MPM) is a rare and aggressive neoplasm deriving from the pleural mesothelial lining. More than 80% of cases are related to previous professional asbestos exposure, with its worldwide incidence being expected to increase in the future. Despite the modest clinical benefit of a multimodality treatment approach including

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surgery, combination chemotherapy and radiation, prognosis remains grim with poor overall survival. For the vast majority of MPM patients ineligible for potentially curative surgery at diagnosis, systemic chemotherapy is the best option to improve survival. The only treatment with level-one level of evidence for improving clinical outcome is the regimen consisting of a platinum doublet with an antifolate. It is possible that standard cytotoxic therapies for MPM have reached a therapeutic plateau, and new approaches based on deregulated pathways and targeted therapies are required to improve survival of MPM patients. We have had a long-standing interest in the role of CD26 in cancer biology and its suitability as a novel therapeutic target in selected neoplasms. Our previous work demonstrated that CD26 is preferentially expressed in MPM cells but not in normal mesothelial cells, and suggested that CD26 expression level correlates with clinical outcomes. More recently, we showed that the CD26-positive population of $CD24^{+}CD9^{+}$ MPM cells exhibits cancer stem cell features. We also reported robust *in vivo* data on the anti-tumor activity of anti-CD26 monoclonal antibody in mouse xenograft models. We herein review significant novel findings and the early clinical development of a CD26-targeted therapy for MPM, advances that can lead to a more hopeful future for MPM patients.

Keywords: malignant pleural mesothelioma, CD26, DPPIV, YS110

INTRODUCTION

Malignant pleural mesothelioma (MPM) is a rare and aggressive neoplasm arising from the pleural mesothelial lining (Kondola et al., 2016), with more than 80% of cases being related to previous occupational asbestos exposure (Myers, 2012; Shersher and Liptay, 2013). Peak incidence of MPM has been reached in the United States, while its incidence is predicted to increase further in the next decades in developing countries where asbestos has not yet been prohibited (Myers, 2012; Shersher and Liptay, 2013). Worldwide, about 80% of mesothelioma deaths occur in ten countries, with Japan, United Sates and United Kingdom being in the top three (van Meerbeeck and Damhuis, 2011; Robinson, 2012). Despite the modest clinical benefit of a multimodality treatment approach including surgery, combination chemotherapy and radiation, prognosis remains grim with poor overall survival (Abdel-Rahman and Kelany, 2015; Maggioni et al., 2016). For the vast majority of MPM patients ineligible for potentially curative surgery at diagnosis, systemic chemotherapy is the best option to improve survival (Kondola et al., 2016).

The only treatment with level-one level of evidence for improving clinical outcome is the regimen consisting of a platinum doublet with an antifolate (Haas and Sterman, 2013). With this combined chemotherapy, patients with good performance status have a median overall survival (OS) of approximately 1 year, and a median progression free survival (PFS) of less than 6 months (van Meerbeeck et al., 2005). There is no standard second line treatment with documented ability to prolong survival. Due to the lack of efficacy of conventional treatments, novel therapeutic strategies are urgently needed to improve outcomes of MPM (Astoul et al., 2012; Haas and Sterman, 2013; Kondola et al., 2016).

Successful treatment of MPM depends on an in-depth understanding of the biology of mesothelioma. Our group has had a long-standing interest in the role of CD26 in cancer biology and its suitability as a novel therapeutic target in selected neoplasms (Thompson et al., 2007; Havre et al., 2008; Ohnuma and Morimoto, 2013). CD26 is a 110-kDa, type II transmembrane glycoprotein with known dipeptidyl peptidase IV (DPPIV, EC 3.4.14.5) activity in its extracellular domain and is capable of cleaving N-terminal dipeptides with either L-proline or L-alanine at the penultimate position (Ohnuma et al., 2008a). CD26 activity is dependent on cell type and the microenvironment factors that can influence its multiple biological roles (Thompson et al., 2007; Ohnuma et al., 2011). Although CD26 expression is enhanced following activation of resting T cells, $CD4^+$ $CD26^{high}$ T cells respond maximally to recall antigens such as tetanus toxoid (Morimoto et al., 1989; Dang et al., 1990c). Cross-linking of CD26 with CD3 by solid-phase immobilized monoclonal antibodies (mAbs) can induce T-cell costimulation and IL-2 production by $CD26^+$ T cells (Dang et al., 1990a; Morimoto and Schlossman, 1998). In addition, anti-CD26 antibody treatment of T cells enhances activation associated with tyrosine phosphorylation of signaling molecules such as CD3ζ and $p56^{lck}$, while its DPPIV enzyme activity is required for CD26-mediated T cell costimulation (Tanaka et al., 1993; Hegen et al., 1997; Ishii et al., 2001). CD26 therefore has an important role in T cell biology and overall immune function (Morimoto and Schlossman, 1998; Ohnuma et al., 2008a).

CD26 is also expressed on various tumors including MPM, renal cell carcinoma (RCC), colorectal cancer, hepatocellular carcinoma, lung cancer, prostate cancer, gastrointestinal stromal tumor, thyroid cancer, and hematologic malignancies such as T-anaplastic large cell lymphoma, T-lymphoblastic lymphoma and T-acute lymphoblastic leukemia (Thompson et al., 2007; Havre et al., 2008; Ohnuma and Morimoto, 2013). In addition to its

expression in cancer cells, CD26 regulates topoisomerase IIα level in hematological malignancies, affecting sensitivity to doxorubicin and etoposide (Yamochi et al., 2005). Moreover, CD26 itself appears to be a novel therapeutic target, and anti-CD26 mAb treatment resulted in both *in vitro* and *in vivo* anti-tumor activity against selected tumor types including MPM (Ho et al., 2001; Inamoto et al., 2006; Inamoto et al., 2007). Our previous work demonstrated that CD26 is preferentially expressed in MPM cells but not in normal mesothelial cells, and suggested that CD26 expression level correlates with clinical outcomes, while being a potential therapeutic target in MPM (Amatya et al., 2011; Aoe et al., 2012). We herein review mainly our significant novel findings and the early clinical development of a CD26-targeted therapy for MPM.

MOLECULAR CHARACTERIZATION OF CD26

In 1979, a large molecular weight complex composed of adenosine deaminase (ADA) activity was found to be an ADA-binding protein (ADBP), also known as adenosine deaminase complexing protein-2 (ADCP2) (Daddona and Kelley, 1979). In 1992, this ADBP or ADCP2 is determined to be identical to CD26, a T-cell activation molecule and a 110-kD glycoprotein that is present also on epithelial cells of various tissues including the liver, kidney, and intestine (Hopsu-Havu and Glenner, 1966; Marguet et al., 1992; Tanaka et al., 1992). The human CD26 cDNA contains a 3,465 base pair (bp) open reading frame that encodes a 766 amino acid protein. The human CD26 amino acid sequence has 85% amino acid identity with the mouse and rat CD26, and 37% amino acid identity with *D. melanogaster* (Marguet et al., 1992; Tanaka et al., 1992). The 5'-flanking region does not contain a TATA box or CAAT box, commonly found in housekeeping genes (Bohm et al., 1995). CD26 does contain a 300 bp G-C rich region with potential binding sites for NF-κB, AP2, or Sp1 (Erickson et al., 2000b). CD26 expression is activated by interferons (IFNs) and retinoic acid in chronic lymphocytic leukemia via Stat1α and the GAS (IFN-γ activaton site) response element (TTCnnnGAA located at bp -35 to -27) in the CD26 promoter (Bauvois et al., 2000). A hepatocyte nuclear factor 1 binding site at position -150 to -131 of the CD26 gene regulates CD26 expression in human intestinal (Caco-2) and hepatic epithelial (HepG2) cell lines (Erickson et al., 1999; Erickson et al., 2000a).

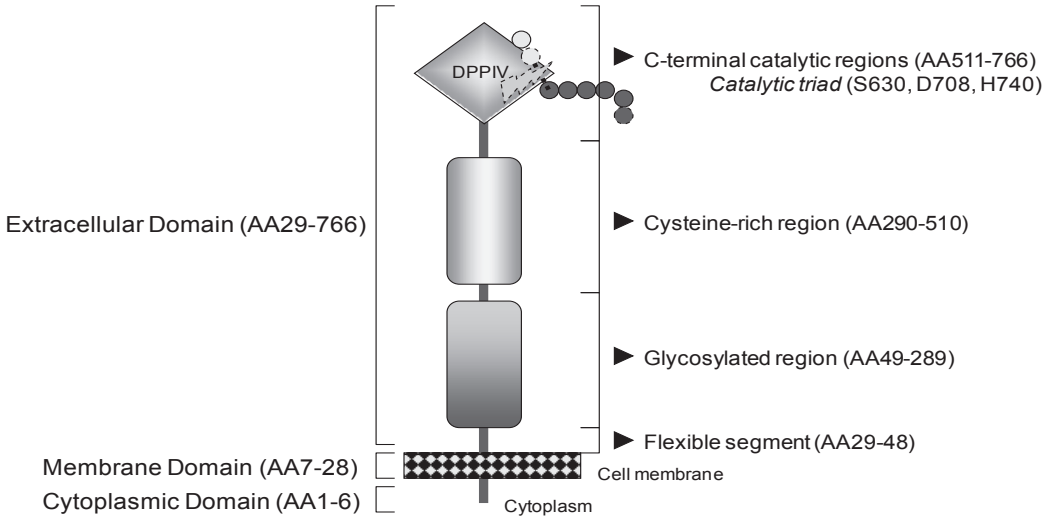

Figure 1. Schematic diagrams of the amino acids of human CD26.
Human CD26 cDNA is composed of 2,301 base pairs, translated to a 766 amino acid protein. DDPIV catalyzes the hydrolysis of N-terminal dipeptides from polypeptides with proline or alanine in the penultimate position. See text for further details.

CD26 is a multifunctional membrane-bound glycoprotein present as a homodimer on the surface of most cell types (Chien et al., 2004; Ohnuma et al., 2008a). Human CD26 is composed of 766 amino acids (Figure 1), including a short cytoplasmic domain of 6 amino acids, a transmembrane region of 24 amino acids, and an extracellular domain with dipeptidyl peptidase activity which selectively removes the N-terminal dipeptide from peptides with proline or alanine at the penultimate position (Heins et al., 1998; Ohnuma et al., 2008a). Analysis of single amino acid point mutations in the β-propeller motif identified Glu205 and Glu206 to be essential for DPPIV enzyme activity, and the central tunnel and α/β-hydrolase domains both participate in DPPIV inhibitor binding (David et al., 1993; Abbott et al., 2000; Rasmussen et al., 2003). CD26/DPPIV was initially considered to cleave peptides only after a proline or alanine residue, but its substrates are now known include hydroxyproline, serine, glycine, valine, threonine, and leucine (Yaron and Naider, 1993; Abbott et al., 2000; Bjelke et al., 2006). CD26 binds to caveolin-1 on antigen presenting cells (APC), and residues 201 to 211 of CD26 along with the serine catalytic site at residue 630, which constitute a pocket structure of CD26/DPPIV, contribute to its binding to the caveolin-1 scaffolding domain (Ohnuma et al., 2004). This region in CD26 contains a caveolin-binding domain ($\Phi X\Phi XXXX\Phi XX\Phi$; Φ and *X* depict aromatic residue and any amino acid, respectively), specifically WVYEEEVFSAY in CD26. These observations strongly support the notion that DPPIV enzyme activity is necessary for CD26-mediated T-cell costimulatory activation, as demonstrated

in our previous work using CD26-targeted mAbs (Tanaka et al., 1993; Morimoto and Schlossman, 1998). Single amino acid point mutation analysis showed that His750 residue is responsible for dimerization (Chien et al., 2004), which is required for T cell costimulation signaling (Ohnuma et al., 2007).

A soluble form of the protein with conserved DPPIV enzyme activity (sCD26 or sDPPIV) is present in the serum and other body fluids, presumably as a result of shedding or secretion from different cell types (Nagatsu et al., 1968; Iwaki-Egawa et al., 1998; Cordero et al., 2009; Rohrborn et al., 2014). The soluble form lacks the transmembrane region and cytoplasmic residues, as it begins at the 39th amino acid residue (Iwaki-Egawa et al., 1998). This form also exists as a dimer and has been detected in seminal fluid as a larger oligomer (>900 kDa) (Iwaki-Egwa et al., 1998). DPPIV enzyme activity is involved in the activation/inactivation of a number of chemokines and cytokines to regulate immune and endocrinological functions as well as cancer cell biology. (De Meester et al., 2000; Thomspn et al., 2007; Havre et al., 2008; Ohnuma et al., 2008a; Ohnuma and Morimoto, 2013). The clinical significance of sCD26 or sDPPIV in MPM patients has been shown in our recent study, which included 80 MPM patients, 79 subjects with past asbestos exposure (SPE), and 134 patients with other benign pleural diseases (OPD) as a control group (Fujimoto et al., 2014). Serum sCD26 levels and sDPPIV enzyme activity in patients with MPM were significantly decreased compared with those in the SPE group. The level of serum sCD26 was significantly decreased in patients with advanced stages of MPM compared with those with earlier stages. 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. The sCD26 levels in the pleural fluid of MPM patients with an epithelioid subtype were significantly increased compared with the OPD cohort. Moreover, DPPIV enzyme activity in the pleural fluid of patients with MPM with an epithelioid subtype was significantly increased compared with that in the OPD cohort. Patients with MPM who had lower specific DPPIV activity, determined as the ratio of DPPIV activity to sCD26 protein quantification, exhibited significantly prolonged survival compared with those with higher specific DPPIV activity. Serum sCD26 and DPPIV enzyme activity appear to be useful biomarkers for differentiating patients with MPM from SPE. In addition, the sCD26 level and DPPIV enzyme activity in pleural fluid appear to be biomarkers in patients with the MPM epithelioid subtype. DPPIV activity in serum or pleural fluid may also be predictive markers for the prognosis of MPM patients (Fujimoto et al., 2014).

CD26 AND IMMUNE FUNCTION

In human peripheral blood, CD26 is found on $CD4^+$ T memory cells and $CD8^+$ effector/memory T cells (Morimoto et al., 1989; Dang et al., 1990c; Hatano et al., 2013). It has been reported that 0-5% of freshly isolated $CD20^+$ B cells do express the CD26 antigen (Buhling et al., 1995). Following stimulation with PMA (phorbol 12-Myristate 13-acetate) or *Streptococcus aureus* protein, the fraction of CD26-positive cells increased to 51% (Morimoto and Schlossman, 1998; Fujimaki et al., 2008). Meanwhile, CD26 is not expressed or is found only at low levels on monocytes of healthy adults (Stohlawetz et al., 1998; Ohnuma et al., 2001). Flow cytometric analysis of dendritic antigen-presenting cells (DC) generated from peripheral blood of normal donors in the presence of granulocyte/macrophage colony-stimulating factor and IL-4 revealed intermediate levels of CD26 expression during a 2-week culture period (Alijagic et al., 1995). Only a small fraction of peripheral NK cells was found to express CD26 (Buhling et al., 1994).

CD26 is a co-stimulatory molecule for T-cell signal transduction. While CD26 expression is enhanced following activation of resting T cells, $CD4^+CD26^{high}$ T cells respond maximally to recall antigens such as tetanus toxoid (Dang et al., 1990c; Morimoto and Schlossman, 1998). Moreover, we have previously reported that effector CD26-mediated costimulatory activity is exerted via its DPPIV enzymatic activity (Tanaka et al., 1993). In addition, $CD4^+$ T cells with *in vitro* transendothelial migratory capacity appear to express high CD26 (Masuyama et al., 1992), and patients with autoimmune diseases such as multiple sclerosis, Grave's disease, and rheumatoid arthritis have been found to have increased numbers of $CD4^+CD26^+$ T cells in inflamed tissues as well as in their peripheral blood, with enhancement of CD26 expression in these autoimmune diseases correlating with disease activity (Ohnuma et al., 2011). Moreover, $CD26^{high}CD8^+$ T cells in humans belong to early effector memory T cells, and $CD26^{high}CD8^+$ T cells exhibit increased expression of granzyme B, TNF-α, IFN-γ and Fas ligand, and exert cytotoxic effect with CD26-mediated costimulation (Hatano et al., 2013).

The cytoplasmic tail of CD26 is responsible for T-cell costimulation induced by anti-CD3 plus caveolin-1 (Ohnuma et al., 2007). Our work found that CARMA1 binds to the cytoplasmic tail of dimeric CD26, and that a PDZ domain in CARMA1 is necessary for binding to CD26 (Ohnuma et al., 2007). Following its phosphorylation, CARMA1 functions as a signaling intermediate downstream of PKCθ (protein kinase θ) and upstream of IKK (IκB kinase) in

the TCR (T cell receptor) signaling transduction pathway, which leads eventually to NF-κB activation. Dimeric CD26, but not monomeric CD26, binds to CARMA1 (Ohnuma et al., 2007). The enzymatic pocket structure of the DPPIV catalytic site is necessary for binding of CD26 to caveolin-1, leading to the upregulation of CD86 expression on APC (Ohnuma et al., 2005). Dimerization of CD26 is therefore not only necessary for binding to caveolin-1, but also serves as a scaffolding structure for the cytoplasmic signaling molecule CARMA1. Overall, CD26 ligation by caveolin-1 on APC recruits CD26-interacting CARMA1 to lipid rafts, resulting in the formation of a CARMA1-Bcl10-MALT1-IKK complex, and this membrane-associated Bcl10 complex then activates IKK through ubiquitination of NEMO (NF-κB essential modulator) (Ohnuma et al., 2008b).

We recently reported that CD3/CD26 costimulation induces the development of a population of human type 1 regulatory T (T_{reg}) cells from $CD4^+$ T cells with high level of IL-10 production and lymphocyte-activation gene 3 (LAG3) expression (Hatano et al., 2015). Other investigators have also reported that the $CD26^+CD39^-$ T_{reg} subset among $CD4^+$ T_{reg} exhibits high level of IL-10 expression (Hua et al., 2015). These findings strongly suggest that a specific subset of $CD26^+$ T cells plays a role in immune checkpoint system, and that the CD26 molecule may be a novel target for a therapeutic approach involving immune checkpoint blockade.

EXPRESSION OF CD26 IN MPM

Our previous work analyzing extracellular matrix interactions and intracellular signaling events demonstrated that the malignant mesothelial cell line JMN expresses CD26 (Dang et al., 1990b). Our recent in-depth studies of CD26 expression in MPM revealed that CD26 is preferentially expressed in MPM cells but not in normal mesothelial cells (Amatya et al., 2011; Aoe et al., 2012). Tissue samples from eighty one epithelioid (49 differentiated and 32 less differentiated), 34 sarcomatoid, 19 biphasic mesothelioma patients and 8 mesothelioma cell lines were examined via immunohistochemistry for membranous and cytoplasmic expression. Cytoplasmic expression of CD26 was observed in all histological types of mesothelioma, being found in 65 of 81 (80%) of epithelioid mesotheliomas, and 17 of 18 (95%) of epithelioid component of biphasic mesothelioma. Meanwhile, membranous expression of CD26 was not found in sarcomatoid mesothelioma or sarcomatoid component

of biphasic mesothelioma (Figure 2). Moreover, 7 out of 8 mesothelioma cell lines (MESO1, MESO4, HMMME, H226, H2452, H28, and H2052), including sarcomatoid types, exhibited CD26 expression, with the MSTO-211H (MSTO) mesothelioma cell line having no expression of CD26 (Amatya et al., 2011). These same 7 mesothelioma cell lines also demonstrated membranous expression of CD26 in cellblock preparation.

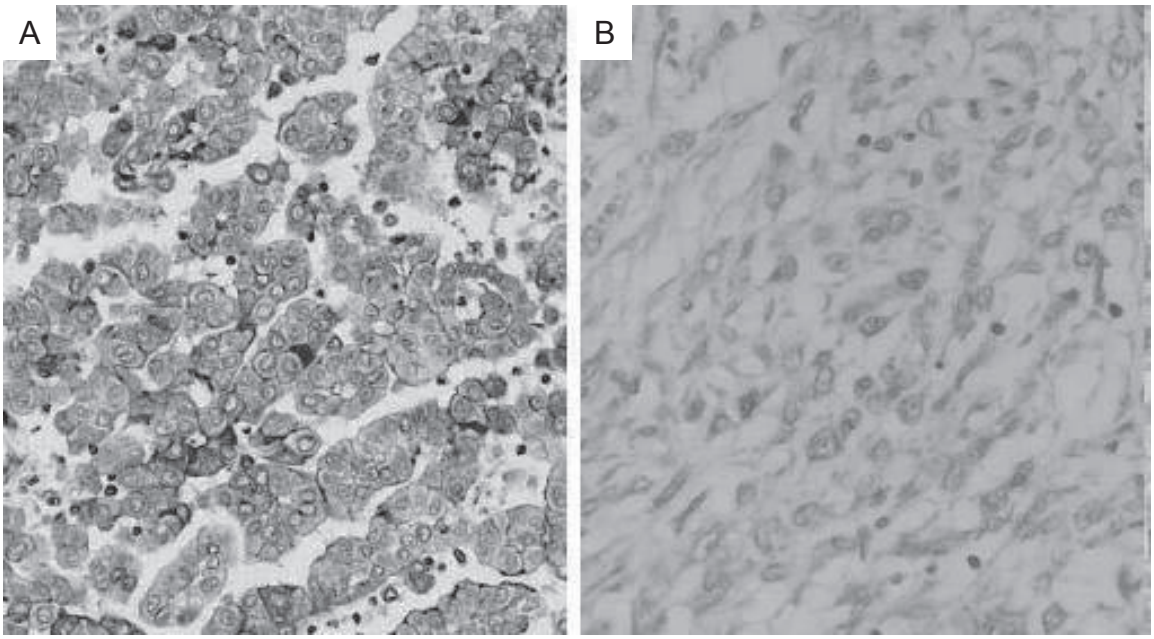

Figure 2. Immunohistochemical staining of MPM with anti-CD26.
Representative serial sections of resected specimens of MPM.
(A) Epithelioid mesothelioma for anti-CD26 immunohistochemistry, showing membranous expression of CD26.
(B) Sarcomatoid mesothelioma for anti-CD26 immunohistochemistry, showing only cytoplasmic expression with inconspicuous membranous expression of CD26.
This figure is reprinted with permission from K Aoe *et al*., *Clin Cancer Res* 18:147-1456, 2012.

CLINICAL RELEVANCE OF CD26 EXPRESSION AND MPM

To explore the role of CD26 for MPM in the clinical setting as a prognostic and therapeutic biomarker, we evaluated 79 MPM cases for CD26 expression and clinical outcome (Aoe et al., 2012). Fifty-eight patients with mesothelioma (73.4%) expressed CD26 on the mesothelioma cell membrane. The majority of patients with epithelioid and biphasic type of mesothelioma expressed CD26 on the mesothelioma cell membrane, whereas none of the patients with the sarcomatoid type did. It should be noted that diffuse staining

for CD26 in the cytoplasm of the mesothelioma cells was observed in all patient samples, even in patients with the sarcomatoid type (Figure 2). We next evaluated the prognostic significance of CD26 membrane expression and other clinicopathological factors in those patients (Aoe et al., 2012). The median survival time (MST) for all mesothelioma patients in the current study was 13.9 months, with the 1-year survival rate being 58.9%. Older age, sarcomatoid histology, advanced stage, absence of extrapleural pneumonectomy (EPP), absence of chemotherapy, and best supportive care were factors associated with poor prognosis. However, no significant relationship between membranous CD26 expression in malignant mesothelioma and survival was observed (MST, 15.0 *vs*. 10.8 months, P=0.1384). Potential relationship between CD26 expression and chemotherapy response and survival was also analyzed. Of the 56 patients treated with chemotherapy, 15 patients had adjuvant chemotherapy, and one patient died prior to evaluation of response to chemotherapy. Therefore, we evaluated the relationship between membranous CD26 expression and response to chemotherapy for 40 patients. The response rate to chemotherapy was marginally associated with membranous CD26 expression (P=0.053). There was a trend toward an association between higher level of CD26 membrane expression and better response to chemotherapy. Univariate analysis of survival time for patients who received chemotherapy demonstrated that membranous CD26 expression was a significant factor associated with better survival (MST, 18.6 *vs*. 10.7 months, P=0.0083). In addition, other clinicopathological factors, including epithelioid histology type, lower stage, EPP or treatment with pemetrexed were also observed to be associated with overall survival. Multivariate analysis for response to chemotherapy showed that epithelioid histology (P=0.016) and EPP (P=0.005) had independent prognostic significance. Membranous CD26 expression showed a hazard ratio of 1.947, but without statistically significant value (P=0.067). Taken together, we concluded that the CD26 molecule is a reliable biomarker for predicting potential therapeutic outcome following chemotherapy (Aoe et al., 2012).

MPM Cancer Cell Biology of CD26

Since CD26 membrane expression on mesothelioma cells appears to be a predictive marker of response to chemotherapy, we next attempted to characterize in details the CD26-positive cells of mesothelioma cell lines. For this purpose we sorted naturally occurring CD26-positive MESO1

mesothelioma cells and subdivided them into CD26-positive and CD26-negative cells. Such cells were subsequently cultured for the indicated days. $CD26^{high}$ cells always exhibited higher level of proliferative activity than CD26-negative cells. Similarly, $CD26^{high}$ cells from other naturally occurring CD26-positive mesothelioma cell lines such as H226 and H28 cell lines consistently had higher level of proliferation than CD26-negative cells from the same cell lines. Moreover, CD26-knockdown of MESO1, JMN and H28 cells resulted in reduced proliferation as compared to wild type CD26-positive MESO-1, JMN and H28 cells, respectively. These results hence suggest that CD26-positive mesothelioma cells have generally robust growth activity (Aoe et al., 2012). Since highly proliferative cells are typically sensitive to chemotherapeutic agents, our present data support the notion that mesothelioma patients with tumors expressing high level of CD26 generally have chemosensitive disease, as discussed in the preceding section.

To extend our previous studies on the relationship between high proliferative activity and invasiveness and high expression level of CD26, we analyzed the signaling events downstream of CD26 in MPM. With the proximal signaling events associated with the cytoplasmic region of CD26 being previously shown in normal human T lymphocytes (Ohnuma et al., 2007), it is conceivable that similar CD26-mediated proximal events may play a role in MPM cell biology. To define the crucial role of the CD26 cytoplasmic region in regulating migratory, invasive or proliferative activity of MPM cells, we used a mutant construct of CD26 in which its cytoplasmic region was replaced with that of human CD10 (CD26-CD10 chimeric receptor), which was shown to abrogate CD26-mediated costimulation in T cells (Ohnuma et al., 2007). CD10, as is the case with CD26, is a type II transmembrane glycoprotein with a relatively short cytoplasmic tail containing signal sequence that has an expected membrane topology similar to CD26 (Ogata et al., 1989; Maguer-Satta et al., 2011). We transfected CD26-negative parental MSTO cells with full-length human CD26 (CD26WT) or CD26-CD10 chimeric receptor (CD26/10Chi) to establish MSTO-CD26WT or MSTO-CD26/10Chi, respectively (Yamamoto et al., 2014). A significant increase in migration was observed in MSTO-CD26WT as compared to MSTO-Mock (vector alone) or MSTO-CD26/10Chi (Figure 3A). Similarly, an increase in invasion was also observed in MSTO-CD26WT as compared to MSTO-Mock or MSTO-CD26/10Chi (Figure 3B). Moreover, a significant increase in colony formation was observed in MSTO-CD26WT as compared

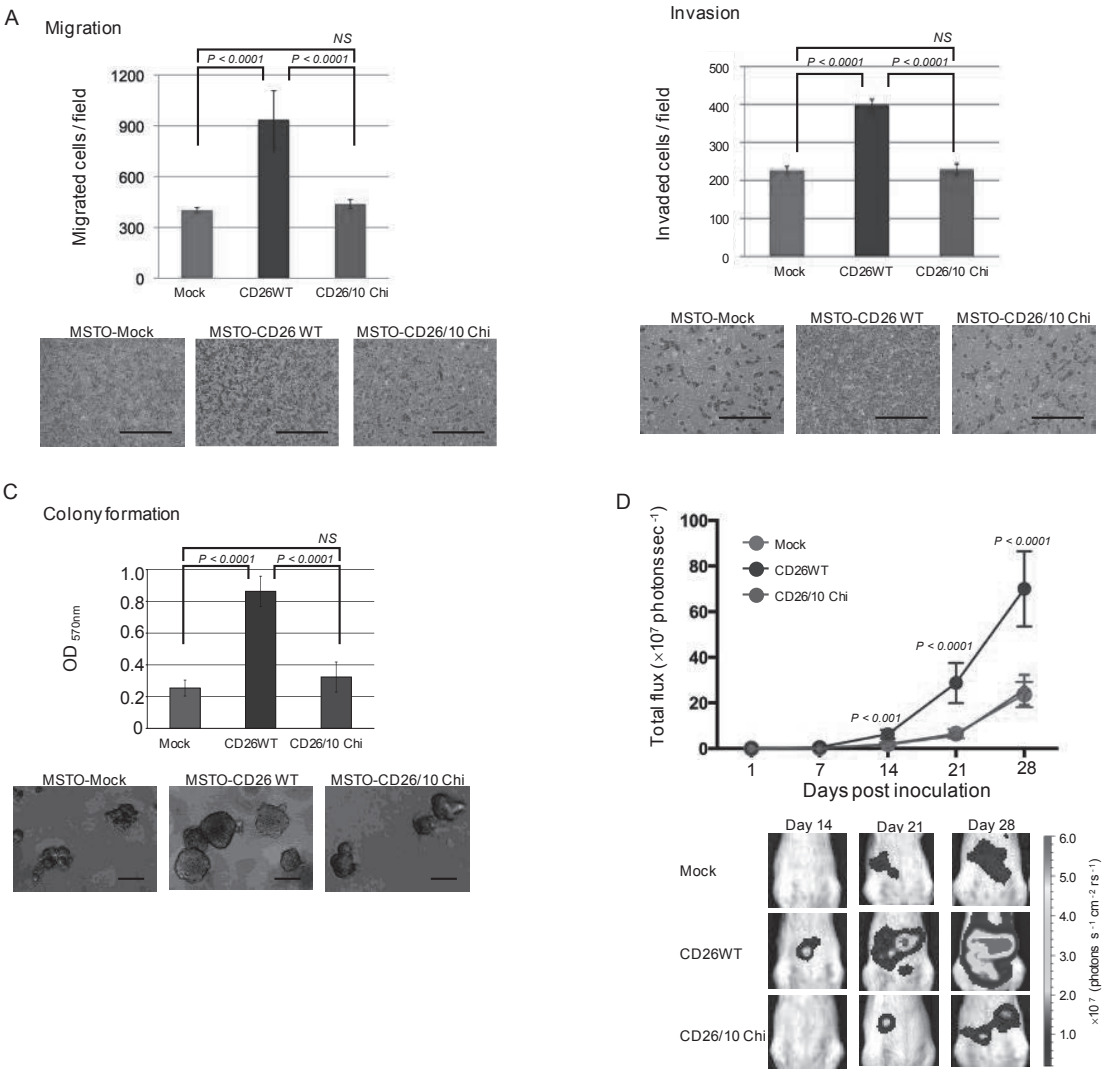

Figure 3. The cytoplasmic region of CD26 is required for cell migration, invasion and colony formation.

(A) Cells were seeded on top of a Boyden chamber. The number of cells that migrated through the uncoated filter in the lower chamber was counted. The mean number of cells per field was determined from 5 fields per filter (mean ± SEM; n=5 experiments with triplicates). A significant increase of MSTO-CD26WT is indicated as $P<0.0001$ (*v.s.* MSTO-Mock or MSTO-CD26/10Chi), as calculated by ANOVA with Tukey-Kramer *post-hoc* test. *NS* denotes 'not significant'. Representative microphotographs of cells migrating through the filter were shown in the lower panels (crystal violet staining). Scale bars indicate 200μm. (B) Cells were seeded on top of Matrigel-coated chamber inserts. The number of cells that invaded through the Matrigel in the lower chamber was counted. The mean number of cells per field was determined from 5 fields per filter (mean ± SEM; n=5 experiments with triplicates). A significant increase of MSTO-CD26WT is indicated as $P<0.0001$ (*v.s.* MSTO-Mock or MSTO-CD26/10Chi), as calculated by ANOVA with Tukey-Kramer *post-hoc* test. *NS* denotes 'not significant'. Representative microphotographs of cells invading through the filter were shown in the lower panels (crystal violet staining). Scale bar indicates 200μm.

(C) Cells were plated in a cell suspension agar matrix between layers of base agar matrix. After 1 week, the agar matrix was solubilized, and cells were stained with MTT solution. Absorbance at 570nm was measured (mean ± SEM; n=5 experiments with triplicates). A significant increase of MSTO-CD26WT is indicated as $P<0.0001$ (*v.s.* MSTO-Mock or MSTO-CD26/10Chi), as calculated by ANOVA with Tukey-Kramer *post-hoc* test. *NS* denotes 'not significant'. Representative microphotographs of cells grown in soft agar just prior to solubilization to indicate cell size and morphology were shown in the lower panels (phase-contrast imaging). Original magnification, ×8. Scale bars indicate 50μm. (D) SCID mice were injected i.p. with 1×10^5 luciferase-expressing MSTO-Mock, MSTO-CD26WT or MSTO-CD26/10Chi cells. Tumor growth was measured by *in vivo* bioluminescence photometry, with imaging data of each cohort being indicated as total flux of photons per second (mean ± SEM; n=20). A significant increase of MSTO-CD26WT is indicated as $P<0.0001$ (*v.s.* MSTO-Mock or MSTO-CD26/10Chi), as calculated by ANOVA with Tukey-Kramer *post-hoc* test. Representative optical bioluminescence imaging of each cohort mice was shown with intensity of luminescence as heat maps in the lower panels. This figure is reprinted with permission from J Yamamoto *et al*., *Br J Cancer* 110:2232-2245, 2014.

to MSTO-Mock or MSTO-CD26/10Chi (Figure 3C). To extend the above *in vitro* results to *in vivo* experimentation, we performed a cell growth assay using xenograft mice. A significant increase in *in vivo* tumor growth was observed with MSTO-CD26WT as compared to MSTO-Mock or MSTO-CD26/10Chi (Figure 3D). These results suggest that the cytoplasmic region of CD26 is important for CD26 function in such MPM biological processes as cell migration, invasion and anchorage-independent cell growth as well as *in vivo* tumor growth, and clinical benefit may be obtained by targeting the CD26 molecule in MPM therapy.

CD26 AND CANCER STEM CELLS IN MPM

The putative existence of cancer stem cell (CSC) is a widely accepted notion at the present time (Reya et al., 2001). CSC proliferates in an asymmetric cell division-like manner, exhibits various stem cell signatures, and is believed to be a potential reason for resistance to therapy. Recent studies have demonstrated the existence of CSCs not only in hematologic malignancies but also solid tumors (Frank et al., 2010; Wang and Dick, 2005). Our recent work identified SP (side-population), CD9, CD24, and CD26 as MPM CSC markers that correlated with primary stem cell signatures (Ghani et al., 2011). SP cells of H226 and H2452 cell lines, and CD24-positive cells of JMN and H226 cell lines proliferated in an asymmetric cell division-like manner. The expression of CD26 closely correlated with that of CD24 in

sarcomatoid type cell lines. In addition, CD9 and CD24-positive cells displayed a higher potential to generate spheroid colony than negative cells in the stem cell medium. Moreover, these marker-positive cells had a clear tendency to generate larger tumors in mouse transplantation assay (Ghani et al., 2011). We further analyzed the CSC properties of CD24 and CD26-positve MPM cells (Yamazaki et al., 2012). We established RNA interference (RNAi) -knockdown MPM cells and found that these markers correlated significantly with chemoresistance, proliferation, and *in vitro* invasion potential. Interestingly, while MESO1 cells expressed both CD24 and CD26, the presence of each of these two markers was correlated with different CSC property. To distinguish intracellular signals downstream of these cell surface antigens, we performed DNA microarray analysis of $CD24^+$ and $CD24^-$ cells of JMN and H226, $CD24^+CD26^+$ and $CD24^-CD26^-$ cells of MESO1, and control-short hairpin RNA (shRNA) and CD24-shRNA cells of MESO1. We found that several genes related to cancer development and stem cell signatures were differentially regulated. Among the genes up-regulated in the positive cells, insulin-like growth factor binding protein 7 (IGFBP7) was commonly up-regulated in all experiments (fold change, JMN-$CD24^+$, 2.5×; H226-$CD24^+$, 3.3×; MESO1-$CD24^+CD26^+$, 27.3×; MESO1-control/CD24-shRNA, 1.9×). In $CD24^+CD26^+$/$CD24^-CD26^-$ cells and control/$CD24^-$ shRNA cells of MESO-1, IGFBP3 (another member of IGFBP, 7.9× and 5.8×, respectively), a cancer gene Wnt5A (5.2× and 6.3×, respectively), and hematopoietic/lymphoid stem cell antigen CD127 (interleukin 7 receptor, IL7R, 10.5× and 3.0×, respectively) were also significantly up-regulated. The microarray data of these genes were further confirmed by RT-PCR in the CD24/CD26-isolated MESO1 cells. Interestingly, expression of IGFBP3 and IGFBP7 was well-correlated with that of CD26, rather than CD24. Meanwhile, expression of Wnt5A and IL7R was correlated with not only CD24 but also CD26 expression, while their expression was completely absent in the $CD24^-CD26^-$ cells. These data suggest that CD24 and CD26 expression correlated with several cancer and stemness genes. In addition to gene expression profiles, our examination of downstream signaling events relating to IGFBP3 and IGFBP7 showed that phosphorylation of ERK (extracellular signal-regulated kinase) by EGF (epidermal growth factor) stimulation was significantly affected by the expression of CD26, but not CD24. Taken together, our data suggest that CD24 and CD26 differentially regulate the CSC potentials of MPM and could be promising targets for CSC-oriented therapy.

ASSOCIATION OF CD26 AND EXTRACELLULAR MATRIX IN MPM

To further explore the molecular mechanisms involved in the highly proliferative activity of CD26-positive MPM cells, we focused on the interaction between CD26 and CD9, and identified as novel markers for cancer stem cells in malignant mesothelioma (Ghani et al., 2011). We found that CD26 and CD9 co-modulated and coprecipitated with each other in the malignant mesothelioma cell lines MESO1 and MSTO (Okamoto et al., 2014). RNAi study revealed that depletion of CD26 led to increased CD9 expression, while depletion of CD9 resulted in increased CD26 expression. Consistent with these findings was the fact that gene transfer of CD26 into CD26-negative MSTO cells reduced CD9 expression. Cell invasion assay showed that overexpression of CD26 or gene depletion of CD9 led to enhanced invasiveness, while CD26 gene depletion resulted in reduced invasive potential. Furthermore, co-precipitation studies demonstrated an association between CD26 and α5β1 integrin, suggesting that this enhanced invasiveness may be partly mediated by α5β1 integrin. Finally, gene depletion of CD9 resulted in elevated protein levels and tyrosine phosphorylation of FAK (focal adhesion kinase) and Cas-L/NEDD9, which are downstream signaling molecules of β1 integrin, while depletion of CD26 led to a reduction in the levels of these molecules. Collectively, our findings suggest that CD26 potentiates tumor cell invasion through its interaction with α5β1 integrin, and CD9 negatively regulates tumor cell invasion by reducing the level of CD26-α5β1 integrin complex through an inverse correlation between CD9 and CD26 expression (Okamoto et al., 2014).

Along with our previous observation that depletion of CD26 by RNAi resulted in the loss of adhesive property, suggesting that CD26 is a binding protein to the extracellular matrix (ECM), the above observation regarding the CD26-α5β1 integrin complex suggests that CD26 regulates the interaction of MPM cells with the ECM via yet-to-be-determined integrin adhesion molecules. More recently, we found that expression of CD26 upregulates periostin secretion by MPM cells, leading to enhanced MPM cell migratory and invasive activity (Komiya et al., 2014). Periostin is a secreted cell adhesion protein of approximately 90kDa, which shares a homology with the insect cell adhesion molecule fasciclin I (FAS1) (Morra and Moch, 2011). Moreover, we showed that upregulation of periostin expression results from the nuclear translocation of the basic helix-loop-helix transcription factor

Twist1, a process that is mediated by CD26-associated activation of Src phosphorylation. In the clinical setting, periostin upregulation has been reported for many cancer types including MPM, and may potentially be a tumor-enhancing factor (Morra and Moch, 2011). Previous work also suggested that periostin expression in MPM cells may be an independent prognostic factor for overall survival (Schramm et al., 2010). Periostin was shown to be not only a marker of epithelial-mesenchymal transition (EMT), but to be itself an inductor of this phenomenon (Yan and Shao, 2006). Our results hence suggest that CD26 expression is associated with EMT via its regulation of periostin.

We demonstrated that CD9 suppresses cell adhesion by inhibiting CD26-α5β1 integrin complex through its negative regulation of CD26 (Okamoto et al., 2014). These observations show that CD26 regulates the interaction of MPM cells with the ECM via the integrin adhesion molecules. Meanwhile, as described above, we have shown that the cytoplasmic 6 amino acid residues of CD26 are required for regulating migratory, invasive or proliferative activity of MPM cells. To define the molecular details involved in the critical role of the CD26 cytoplasmic region, we used affinity purification and LC-MS/MS (liquid chromatography-tandem mass spectrometry) to identify the proteins that are associated with the CD26 cytoplasmic domain (Yamamoto et al., 2014). In these experiments, membrane fractions of MSTO-Mock, MSTO-CD26WT or MSTO-CD26/10Chi were harvested in native conditions and subjected to affinity purification using anti-CD26 mAb. We demonstrated that the cytoplasmic region of CD26 plays a crucial role in MPM tumor biology through its linkage to somatostain receptor 4 (SSTR4) and SHP-2 protein tyrosine kinase in cell membrane lipid rafts, leading to cytostatic effects in MPM cells without direct association of the ECM to CD26 (Yamamoto et al., 2014).

In view of the findings above, we propose that CD26 forms macromolecular complexes in the cell surface of MPM (Figure 4), and plays a pivotal role in the malignant activity of MPM by connecting periostin and ECM to intracellular signaling events. It is therefore conceivable that targeting CD26 may be a novel and effective therapeutic approach for MPM.

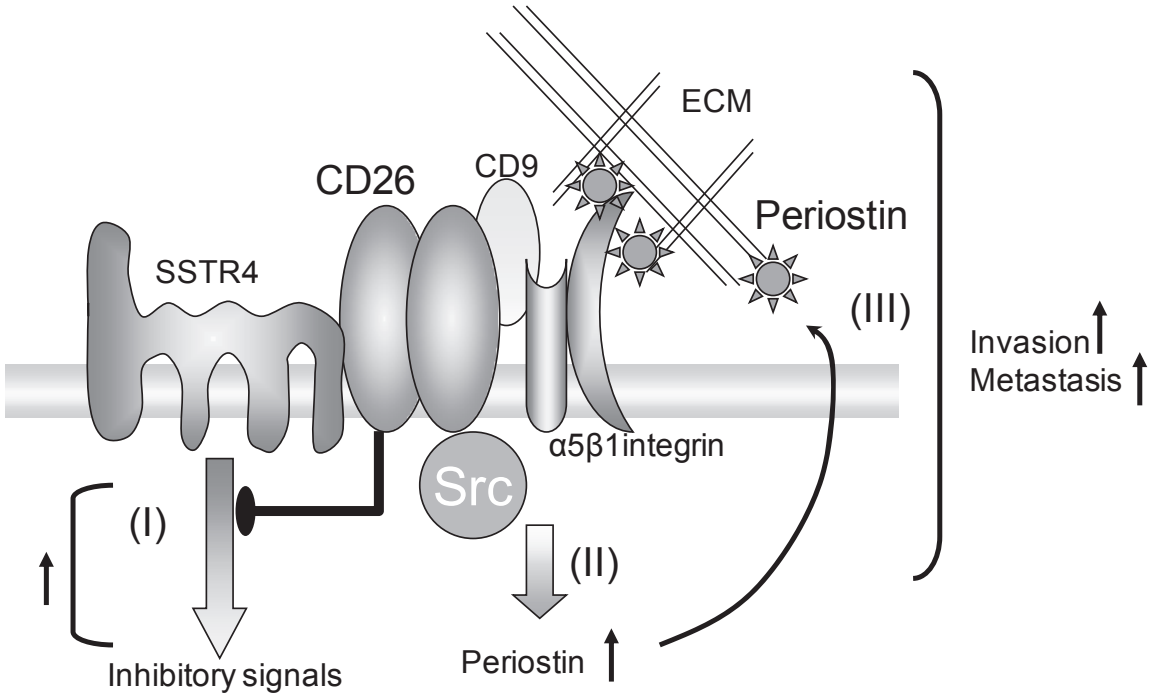

Figure 4. Macromolecular complex associated with cell surface CD26 in MPM.
(I) In CD26-negative MPM cells, SSTR4 mediated inhibitory signaling to suppress cell proliferation and motility. In contrast, by locking the signaling domain of SSTR4 with CD26 association, SSTR4-mediated anti-tumor effects were abrogated, leading to increased cell proliferation and motility in CD26-positive MPM cells.
(II) In addition, CD26 regulated extracellular matrix (ECM)-associated tumor cell behavior in association with integrins and periostin-ECM complex. CD9 suppressed cell invasion and migration by inhibiting the formation of CD26-α5β1 integrin complex. Moreover, expression of CD26 upregulated periostin secretion by MPM cells, leading to enhanced MPM cell migratory and invasive activity. Moreover, we showed that upregulation of periostin expression resulted from the nuclear translocation of the basic helix-loop-helix transcription factor Twist1, a process that was mediated by CD26-associated activation of Src phosphorylation.
(III) Periostin is a secreted cell adhesion protein. The N-terminal region regulates cellular functions by binding to integrins at the plasma membrane of the cells through cell adhesion domain. The C-terminal region of the protein regulates cell–matrix organization and interaction by binding such ECM proteins, leading to increased MPM cell motility. CD26 molecule in MPM plays a pivotal role in connecting ECM to intracellular signaling events associated with cell proliferation and motility.

Preclinical Proof-of-Concept (POC) Study of Anti-CD26 mAb Therapy for MPM

As described in the preceding sections, CD26/DPPIV is expressed at a high level on the surface of malignant mesothelioma cells, but not on cells

derived from normal mesothelial cells. These intriguing findings propelled our development of CD26-targeted therapy for MPM. For this purpose, we had developed a novel humanized anti-CD26 mAb, namely YS110. YS110 is a recombinant DNA-derived humanized mAb that selectively binds with high affinity to the extracellular domain of CD26. The antibody is an $IgG_1\kappa$ with a molecular weight of 144 kDa and was humanized via an *in silico* design based on the amino acid sequence of anti-human CD26 murine mAb (14D10), which inhibited tumor cell growth, migration and invasion, and enhanced survival of mouse xenograft models (Inamoto et al., 2006). YS110 is produced by fermentation in CHO mammalian cell suspension culture with the Glutamine Synthetase Expression System. *In vitro* pharmacologic evaluation of YS110 demonstrated its selective binding to human CD26 on a number of human cancer cell lines and tissues, with no apparent effect on immune activation and no inhibition of DPPIV activity, while exhibiting direct cytotoxic effect on certain human CD26-positive cancer cell lines. Moreover, our *in vitro* data indicated that YS110 induces cell lysis of MPM cells via antibody-dependent cell-mediated cytotoxicity (ADCC) in addition to its direct anti-tumor effect via cyclin-dependent kinase inhibitor (CDKI) $p27^{kip1}$ and $p21^{cip1}$ accumulation (Inamoto et al., 2007; Hayashi et al., 2016). *In vivo* experiments with mouse xenograft models involving human MPM cells demonstrated that YS110 treatment drastically inhibits tumor growth in tumor-bearing mice and reduces formation of metastases, resulting in enhanced survival (Inamoto et al., 2007; Okamoto et al., 2014; Yamamoto et al., 2014). Our data strongly suggest that YS110 may have potential clinical use as a novel cancer therapeutic agent for CD26-positive malignant mesothelioma.

FIRST-IN-HUMAN PHASE I CLINICAL TRIAL OF YS110 FOR MPM

In the POC studies using preclinical models, *in vivo* administration of 14D10 or YS110 resulted in inhibition of tumor cell growth, migration and invasion, and enhanced survival of mouse xenograft models inoculated with RCC or MPM (Inamoto et al., 2006; Inamoto et al., 2007; Okamoto et al., 2014; Yamamoto et al., 2014). In addition to our robust *in vitro* and *in vivo* data on antibody-mediated dose-dependent tumor growth inhibition, YS110 exhibited excellent safety and pharmacological profiles in non-human primate models using single and repeated increasing intravenous doses. Considering

the lack of T cell proliferation and cytokine production *in vitro*, YS110 was therefore considered not to have an agonistic nor activating effect on human CD26-positive lymphocytes. Other key safety findings were obtained from studies involving cymologus monkeys which express YS110-reactive CD26 molecules, with similar tissue distribution profiles and expression levels to human CD26. We therefore conducted the first-in-human (FIH) clinical trial of YS110 for patients with MPM and other CD26-positive solid tumors (Angevin et al., 2015).

Thirty-three heavily pre-treated patients with CD26-positive cancers including 22 malignant mesothelioma, 10 RCC and 1 urothelial carcinoma underwent YS110 administration. A total of 232 infusions (median 3 [range 1-30]) of YS110 were administered across 6 dose levels ranging from 0.1 to 6 mg/kg. Maximum tolerated dose (MTD) was not reached and 2 dose limiting toxicities (DLTs) (1 patient with grade 3 anaphylactic reaction at 1 mg/kg and 1 patient with grade 3 allergic reaction at 2 mg/kg) were reported with complete resolution following dose omission. Subsequent use of systemic steroid prophylaxis and exclusion of patients with significant allergy histories improved safety profile. No dose-dependent adverse events were observed. Blood exposure pharmacokinetics parameters (AUC and C_{max}) increased in proportion with the dose. Cytokines and immunophenotyping assays indicated CD26 target modulation with no occurrence of infectious nor autoimmune disease complications. These results demonstrated that YS110 is tolerable in human subjects.

A secondary objective of this FIH study was to evaluate the potential antitumor activity of YS110 according to RECIST 1.0 criteria (or modified RECIST criteria for mesothelioma). No objective response was achieved in any of the treated patients. However, stable disease as the best overall response was observed in 13 out of the 26 valuable patients on Day 43 of the first cycle (1 at 0.1 mg/kg, 2 at 0.4 mg/kg, 7 at 2.0 mg/kg, 1 at 4.0 mg/kg and 2 at 6.0 mg/kg). Prolonged stabilization was observed in 9 out of 13 stable disease patients who have received a total of 161 (6 to 30) infusions with a median PFS of 32 weeks (12 – 57 weeks). This FIH study conclusively demonstrated that YS110 exhibits a favorable safety profile and substantial clinical activity in heavily pretreated CD26-positive MPM patients who had previously progressed on conventional standard chemotherapies (Angevin et al., 2015).

DPPIV Enzyme Activity and Efficacy of YS110 in MPM

The FIH clinical study of YS110 revealed that an increase in YS110 infusion dose was associated with decreased serum sCD26 level, particularly in cohorts 4-6 (2.0 to 6.0mg/kg), with an approximately 80% decrease in sCD26 level (Angevin et al., 2015). Moreover, since sCD26 level reflects DPPIV enzyme activity in sera (Durinx et al., 2000), similar reduction in DPPIV enzyme activity was observed, again particularly in patients in cohorts 4-6. Although DPPIV inhibitors are clinically used as oral hypoglycemic agents (Drucker and Nauck, 2006), hypoglycemia was not observed during YS110 administration. Of note is the fact that greater than 80% inhibition of serum DPPIV activity was obtained 24 hours after oral administration of clinically available DPPIV inhibitors (drug information published by manufacturers of sitagliptin, vildagliptin, saxagliptin and *etc*.), a level of inhibition comparable to that seen in patients treated with YS110. Our current data would therefore indicate that YS110 therapy is tolerable in the clinical setting.

Recent work has demonstrated the functional role of DPPIV-mediated post-translational modification of chemokines in regulating tumor immunity through its interaction with its substrate CXCL10 (Barreira da Silva et al., 2015). Barreira de Silvia *et al*. use *in vivo* tumor-transplant models to show that DPPIV inhibition reduces tumor growth through the preservation of bioactive CXCL10 in the tumor microenvironment (TME). In the normal physiological state, CXCL10 is rapidly degraded by DPPIV, resulting in decreased recruitment and migration of $CXCR3^+$ T cells into the TME (Figure 5) (Ohnuma et al., 2015). CXCR3 has been shown to be a functional receptor for CXCL10 (Proost et al., 2001). In contrast, DPPIV inhibition enhances tumor rejection by preserving the full-length biologically active form of CXCL10, leading to increased trafficking of $CXCR3^+$ T cells into the TME (Figure 5) (Ohnuma et al., 2015). This anti-tumor response is potentiated in combination with other anti-tumor immunotherapeutic approaches including CpG adjuvant therapy, adoptive T cell transfer therapy and checkpoint blockade therapy (anti-CTLA-4 and anti-PD-1) (Barreira da Silva et al., 2015). In view of these recent findings, data from our current trial showing that serum DPPIV activity was decreased following treatment with YS110 in a dose-dependent manner would suggest that anti-tumor activity via DPPIV inhibition

may constitute yet another mechanism of action for the anti-tumor activity of YS110.

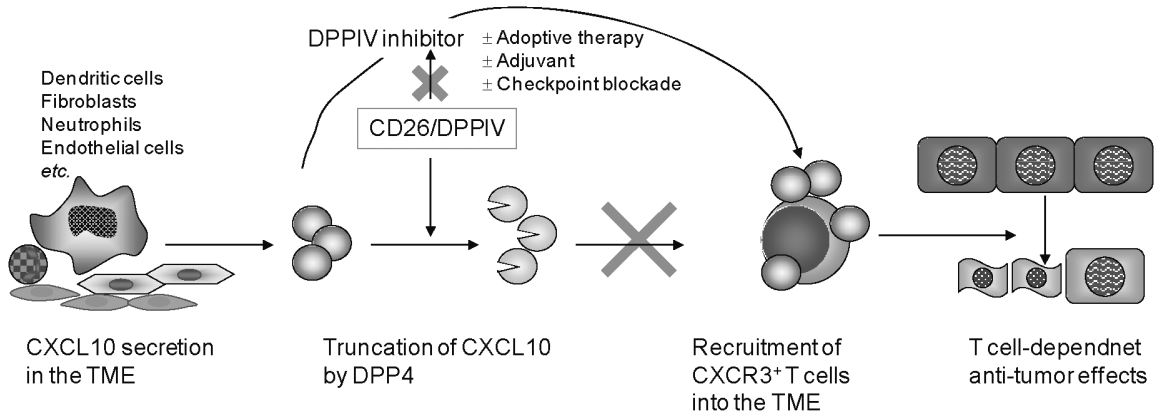

Figure 5. DPPIV inhibition suppresses truncation of its ligand CXCL10, leading to recruitment of $CXCR3^+$ T cells into tumor parenchyma.
In vivo tumor-transplant models showed that DPPIV inhibition reduced tumor growth through the preservation of bioactive CXCL10 in the tumor microenvironment (TME). In the normal physiological state, CXCL10 was rapidly degraded by CD26/DPPIV, resulting in decreased recruitment and migration of $CXCR3^+$ T cells into the tumor parenchyma. In contrast, DPPIV inhibition enhanced tumor rejection by preserving the full-length biologically active form of CXCL10, leading to increased trafficking of $CXCR3^+$ T cells into the tumor parenchyma. This anti-tumor response was potentiated in combination with other anti-tumor immunotherapeutic approaches including CpG adjuvant therapy, adoptive T cell transfer therapy and checkpoint blockade therapy such as anti-CTLA-4 and anti-PD-1.
This figure is reproduced with permission from K Ohnuma *et al.*, *Nat Immunol* 16:791-792, 2015.

MECHANISMS OF ACTION OF YS110 IN MPM

We previously showed that depletion of CD26 by RNAi results in the loss of adhesive property, suggesting that CD26 is a binding protein to the ECM (Inamoto et al., 2007). Moreover, our *in vitro* data indicate that YS110 induces cell lysis of MPM cells via ADCC in addition to its direct anti-tumor effect via CKDI $p27^{kip1}$ accumulation (Inamoto et al., 2007). More recently, we evaluated the direct anti-tumor effect of YS110 against the MPM cell lines H2452 and JMN, and investigated its effects on cell cycle and on the cell cycle regulator molecules (Hayashi et al., 2016). YS110 suppressed the proliferation of H2452 cells by approximately 20% in 48 hours of incubation. Cell cycle analysis demonstrated that the percentage of cells in G2/M phase increased by

8.0% on average following YS110 treatment; in addition, level of the cell cycle regulator $p21^{cip1}$ was increased and cyclin B1 was decreased after YS110 treatment. Inhibitory phosphorylation of both cdc2 (Tyr15) and cdc25C (Ser216) was elevated. Furthermore, activating phosphorylation of p38 MAPK (Thr180/Tyr182) and ERK1/2 (Thr202/Tyr204) was augmented following 24 hours of YS110 treatment. In addition, we investigated the synergistic effects of YS110 and the anti-tumor agent pemetrexed on selected MPM cell lines in both *in vitro* and *in vivo* studies. Pemetrexed rapidly induced CD26 expression on cell surface, and treatment with both YS110 and pemetrexed inhibited *in vivo* tumor growth accompanied by a synergistic reduction in the MIB-1 index.

We also demonstrated that treatment with YS110, which inhibited cancer cell growth, induced nuclear translocation of both cell-surface CD26 and YS110 (Yamada et al., 2009; Yamada et al., 2013). In response to YS110 treatment, CD26 was translocated into the nucleus via caveolin-dependent endocytosis, and interacted with a genomic flanking region of the *POLR2A* gene, a component of RNA polymerase II. This interaction consequently led to transcriptional repression of the *POLR2A* gene, resulting in retarded cancer cell proliferation. Furthermore, impaired nuclear transport of CD26 reversed the *POLR2A* repression induced by YS110 treatment. These findings reveal that nuclear CD26 functions in the regulation of gene expression and tumor growth, while yet another novel mechanism of action of anti-CD26 mAb therapy may involve the regulation of inducible traffic of surface CD26 molecules into the cell nucleus.

Taken together, we summarize the mechanisms of action of YS110 in MPM as follows; (i) a direct cytotoxic effect on certain human CD26-positive cancer cell lines (ADCC), (ii) a direct anti-tumor effect through the induction of cell cycle arrest by induction of $p27^{kip1}$ and $p21^{cip1}$ expression, (iii) following internalization of the CD26-YS110 complexes, an inhibition of invasion and migration of tumor cells by decreased binding to the collagen/fibronectin microenvironment matrix, and (iv) the nuclear translocation of CD26 molecules by internalization of the CD26-YS110 complexes to inhibit proliferation of MPM cells via suppression of *POLR2A* gene expression.

COMPANION DIAGNOSTICS FOR CD26-TARGETING THERAPY

The development of companion diagnostic agents to be used in conjunction with the appropriate therapeutic modalities is essential to maximize therapeutic effectiveness while minimizing associated toxicities. Detection of tumor CD26 expression is critical to determining potential eligibility for treatment with YS110, and it is also important to determine whether CD26 expression on tumors or lymphocytes is affected by anti-CD26 mAb therapy. Immunohistochemical staining of CD26 with the many anti-CD26 mAbs previously developed in our laboratory did not reveal an anti-CD26 mAb that can clearly detect the denatured CD26 molecule in formalin-fixed paraffin-embedded tissues (Dong et al., 1998; Hatano et al., 2014). Meanwhile, testing of several commercially available anti-CD26 mAbs designated as research reagents for immunohistochemical staining indicated that these mAbs could stain the denatured CD26 in formalin-fixed tissues, but not with sufficient clarity. On the other hand, our testing of commercially available anti-CD26 polyclonal antibodies (pAbs) showed that these pAbs exhibited reliable staining pattern and intensity (Amatya et al., 2011; Yamada et al., 2013). However, the disadvantage of pAbs is the potential lot-to-lot variability in staining pattern and intensity, which makes pAbs not the ideal reagents for diagnostic testing of patient specimens in the clinical setting, where consistency and uniformity are required. For this purpose, we recently developed novel anti-human CD26 mAbs that can be used as companion diagnostic reagents suitable for immunohistochemical staining of CD26 in formalin-fixed tissue sections with reliable clarity and intensity (Hatano et al., 2014). Since these mAbs display no cross-reactivity with the therapeutic humanized anti-CD26 mAb, they may be suitable for assays analyzing CD26 expression during or following treatment with the humanized anti-CD26 mAb YS110, with important implications in the clinical setting.

CONCLUSION

While the incidence of MPM continues to increase worldwide, standard chemotherapy does not lead to significant increase in survival. Our increased understanding of the biology and novel therapeutic targets of MPM will pave the way for better treatment options in the future.

Our FIH study showed that YS110 therapy is generally well-tolerated with preliminary evidence of activity in patients with advanced/refractory CD26-expresing cancers including MPM. Our findings also suggest that further clinical development of YS110 such as its use as part of combination therapies with other antineoplastic agents is warranted.

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第2章　核内移行するヒト化抗CD26モノクローナル抗体-TFⅡH阻害剤複合体

山田健人*

1　はじめに

CD26は，細胞膜表面に局在するTリンパ球共刺激分子であり，ヘルパーT細胞のマーカーとして見出された。このCD26は，C末端にセリンプロテアーゼの一つであるDipeptidyl Peptidase-4（DPP-Ⅳ）を持つとともに，別の領域では，細胞外基質であるファイブロネクチンやコラーゲン，あるいはアデノシンデアミナーゼと結合し，さまざまな細胞内情報伝達を調節する働きも有していると考えられている[1)]。正常組織・細胞においては，Tリンパ球以外に，腎近位尿細管上皮，肝毛細胆管，破骨細胞，血管内皮細胞などに発現している。このCD26は，一部の悪性リンパ腫・白血病や腎癌，大腸癌，甲状腺癌に発現していることが知られていたが，最近，悪性中皮腫において85％以上の症例において発現していることが明らかとなった[2~5)]。また中皮腫や大腸癌においては，CD26ががん幹細胞のマーカーであることが報告され[6,7)]，さらにCD26が広範ながんに発現しており，がん細胞の増殖や浸潤，転移にも関与することが明らかとなりつつある[1,8)]。

2　抗CD26モノクローナル抗体とそのヒト化

このようにがん細胞の細胞表面に発現するCD26を分子標的療法の標的分子として捉える抗体療法の開発が試みられた。順天堂大学・森本幾夫博士らが開発したマウス抗ヒトCD26モノクローナル抗体の幾つかのクローンは，ヒトがん細胞株に対して抗腫瘍作用を有すことが判明した[1)]。そこで，ワイズ・セラピューティックス社は，これらの抗体の中から，抗腫瘍効果が強いクローン14D10を選択し，そのcDNAを元にしてAbmaxis *in-silico* Immunization（AISIM）法により，*in silico*でアミノ酸をヒト型に置換することで，親和性を維持したヒト化モノクローナル抗体YS110を開発した。このYS110抗体（ヒトIgG1サブタイプ）は，元となったクローン14D10抗体よりも，ヒトCD26分子に対する親和性が高く，またCD26の細胞外基質への接着阻害能やCD26陽性がん細胞に対する増殖抑制能が亢進していた[3)]。またYS110は，マウスやラットなどのげっ歯類CD26には反応しないが，毒性試験に用いるサルとは交叉反応が認められた。またYS110はCHO細胞により分泌され，精製されるが，安定した高い生産性を示した。

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3　ヒト化抗 CD26 モノクローナル抗体 YS110 の抗腫瘍効果と分子機構（図 1）

通常，抗体療法では，抗体が細胞表面で標的分子と結合した後，抗体の Fc 領域を介した細胞傷害作用が惹起されることで細胞傷害が誘導される。この抗体依存性細胞介在性細胞傷害（antibody-dependent cellular cytotoxicity：ADCC）に加えて，補体依存性細胞障害活性（complement-dependent cytotoxicity：CDC）などの免疫学的機序により，がん細胞が攻撃される。YS110 においても，この ADCC および CDC による抗がん作用が認められた[3]。

一方，培養ヒトがん細胞への YS110 添加あるいは ADCC や CDC が機能しない免疫不全マウス担がんモデルへの YS110 の投与においても，ヒトがん細胞の抗がん作用が判明し，YS110 が

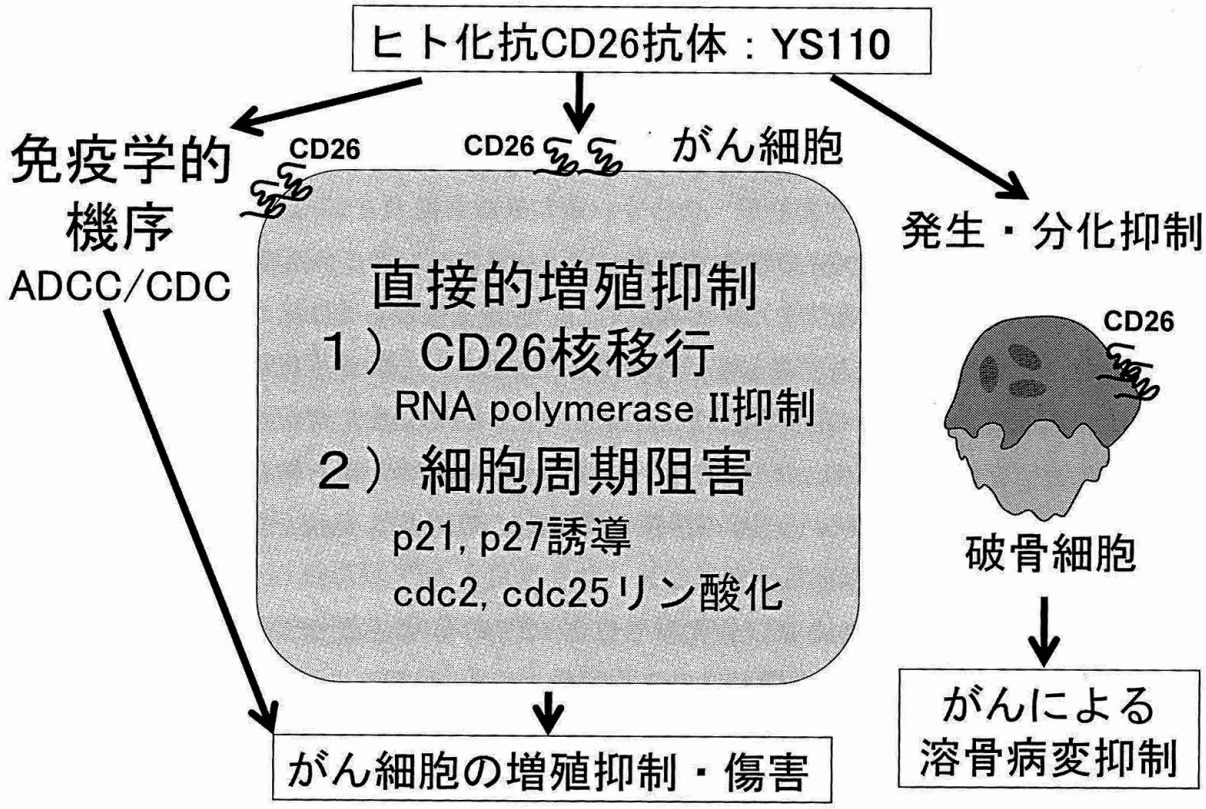

図１　ヒト化抗 CD26 モノクローナル抗体 YS110 のがん細胞への多彩な作用機序

YS110 は，IgG1 サブタイプに属し，抗体依存性細胞介在性細胞傷害（antibody-dependent cellular cytotoxicity：ADCC）や補体依存性細胞障害活性（complement-dependent cytotoxicity：CDC）による免疫学的機序によるがん細胞傷害能を持つ。一方，がん細胞の細胞膜表面に存在する CD26 に結合することにより，がん細胞に対して直接的細胞増殖抑制能を有する。その機序は，一つは，YS110 と結合した CD26 がカベオリン依存性に細胞質に内在化し，さらに核内に移行することで，RNA polymerase Ⅱのサブユニット POLR2A の転写を抑制することによる。また YS110 が細胞膜 CD26 に結合することにより，細胞周期関連分子の発現誘導やリン酸化促進を介して，細胞増殖を抑制する。一方，YS110 は破骨細胞の分化抑制を誘導し，破骨細胞の活性を低下させるため，がんの骨転移における溶骨性病変を抑制する効果を持つ。このように YS110 は，一つの抗体であるが多種にわたる分子機構を介して，がん細胞の増殖浸潤や骨病変の抑制に効果的に寄与しうるユニークな抗体療法となることが期待されている。

直接的にがん細胞に働き，抗腫瘍効果を発揮しうると推測された[3]。その直接的抗がん作用の分子機構の一つは，YS110が細胞膜CD26に結合することにより，細胞周期関連分子p21，p27の発現誘導やcdc2, cdc25のリン酸化促進を介して，細胞増殖を抑制することであった[1, 3]。

いま一つの分子機構が，YS110によるCD26の核移行誘導であった。がん細胞をYS110で処理すると，YS110と結合したCD26がカベオリン依存性に細胞質に内在化し，さらにCD26-YS110複合体が，核内に移行することでRNA polymerase Ⅱのサブユニット POLR2Aの転写を抑制することが判明した[9, 10]。このCD26とYS110の核移行は，CD26を発現する正常細胞（Tリンパ球，血管内皮細胞）では観察されず，がん細胞特異的な現象であった。抗体はもちろんのことであるがCD26にも核移行シグナルはなく，なんらかの核輸送分子と相互作用をすることで核へ移行するものと推測された。このCD26-YS110複合体の核移行は，がん細胞のYS110処理後，30分より観察され，2時間をピークとして4時間後まで観察され，Nystatin処理あるいはカベオリンsiRNA処理により核移行は阻害されたが，Monodansylcadaverineでは阻害されなかったことから，クラスリン依存性ではなくカベオリン依存性のエンドサイトーシスによるものであることが明らかとなった。さらに免疫不全マウスに移植したヒトがん細胞が腫瘍を形成した後，YS110を投与すると *in vivo* においてもCD26およびYS110ががん細胞の核内において観察された[10]。

そこで，核内におけるCD26の機能を探索するためにクロマチン免疫沈降法により，核内CD26が結合しているDNA配列について検討した。核抽出液からCD26を免疫沈降して得られたDNA断片をクローニングして塩基配列を決定したところ，一つが，RNA polymerase Ⅱのサブユニット POLR2A遺伝子の近傍領域であった。そこでPOLR2A遺伝子の調節領域を含むレポーター遺伝子を作製し，CD26の結合性や転写における抑制効果を検討したところ，YS110処理により抗体の量依存性かつCD26の核移行依存性にPOLR2A発現が抑制され，細胞増殖抑制が誘導されることが判明した[10]。

一方，興味深いことにYS110は，骨代謝や骨リモデリングに重要な機能を有する破骨細胞の分化を抑制し，破骨細胞の活性を低下させることで，がんの骨転移における溶骨性病変を抑制する効果を持つことが明らかとなった[11]。

このようにYS110という一つの抗体が，多岐にわたる分子機構を介して，がん細胞の増殖浸潤や骨病変の抑制に効果的に寄与しうることが期待されている（図1）。

このYS110は，サルを用いた安全性試験やGMP準拠の抗体作製を経て，欧州連合における臨床治験申請を行い，2009年からフランスGustave Roussy Instituteを中心として，第Ⅰ相臨床試験が実施され，2015年10月に終了した。その結果，重篤な有害事象は観察されず，中皮腫症例においては，19例中10例がmodified RECIST評価でStable Disease（SD）と判定され，5例が6ヶ月以上SDを継続，1例が3ヶ月以上SDを継続し，YS110単独での治療の有効性を示唆する結果が得られた[12]。

4　YS110へのTFⅡH阻害剤結合による新規ADC（Y-TR1）の開発

CD26陽性のがん細胞をYS110で処理すると，YS110と結合したCD26がカベオリン依存性に細胞質，さらに核内に移行してRNA polymeraseⅡのサブユニットPOLR2Aの転写を抑制することで細胞増殖抑制を惹起することから，YS110へRNA polymeraseⅡの機能阻害分子を結合させることで，核内で効率良く抗がん作用を発揮しうるADCの開発を試みた。RNA polymeraseⅡは，タンパク質をコードするほとんどの遺伝子の転写に必須なきわめて重要な酵素であり，その機能阻害剤としては，アマニチンなどの分子が知られているが，いずれも毒性が強く抗がん剤として患者さんへ全身投与するのは難しいとされてきた。またYS110がRNA polymeraseⅡのサブユニットであるPOLR2A遺伝子の転写を抑制することから，RNA polymeraseⅡ自体の阻害剤ではなく，RNA polymeraseⅡとともに転写に必須とされる基本転写因子群TFⅡHを標的とした阻害剤をYS110へ結合する薬剤として選択した。その中でTriptolideは，RNA polymeraseⅡによる転写イニシエーションに関わる因子TFⅡHのサブユニットXBP/ERCC3に結合することで，DNA依存性ATPase活性を阻害し，結果としてほとんどの遺伝子のRNA polymeraseⅡによる転写を抑制する分子（$C_{20}H_{24}O_6$，分子量360.4）である[13]。またTriptolideはTFⅡH阻害効果は強いものの，難容性であり，薬剤としての使用は難しいと考えられていた。そこで，このTriptolideをYS110に結合させることで，がん細胞特異的に核内に取り込まれることを目論んで，全身での毒性を低く抑えた新規ADCの作製を行った。

このTriptolideにSH基を導入して，S-S結合により化学的に安定な二量体とした。このS-S結合はTCEP還元ゲルを用いて還元され単量体（TR1と命名）としたものをYS110との結合に用いた。YS110にheterobifunctionalリンカーSuccinimidyl 4-[*N*-Maleimidomethyl] Cyclohexane-1-Carboxylate（SMCC）を結合させ，非結合のSMCCをHiTrap脱塩カラムで除去した後，Triptolide誘導体TR1と反応させ，反応産物から非結合TR1を除去してから，抗体を精製し，YS110-Triptolide誘導体TR1結合分子（Y-TR1と命名）とした（図2）。Y-TR1は，まず抗体としてのヒトCD26分子との親和性や結合力価を検討し，その親和性や結合力価にはほとんど低下がないことを確認した。次にY-TR1において，抗体YS110一分子あたりどのくらいのTR1分子が結合しているかを評価した。質量分析器MALDI-TOF massを用いてYS110とY-TR1の質量を測定し，その差をTR1分子量で割ることで，YS110一分子あたり6～7個のTR1分子が結合していることが判明した（図3）。

5　Y-TR1の抗がん作用

この新規ADCであるY-TR1のCD26陽性ヒトがん細胞に対する抗がん作用を検討した。培養細胞としては，CD26陰性中皮腫細胞MSTO-211HにCD26発現ベクターを導入した安定発現細胞株（MSTO-CD26クローン12）と対照としてネオマイシン耐性遺伝子のみを導入した安

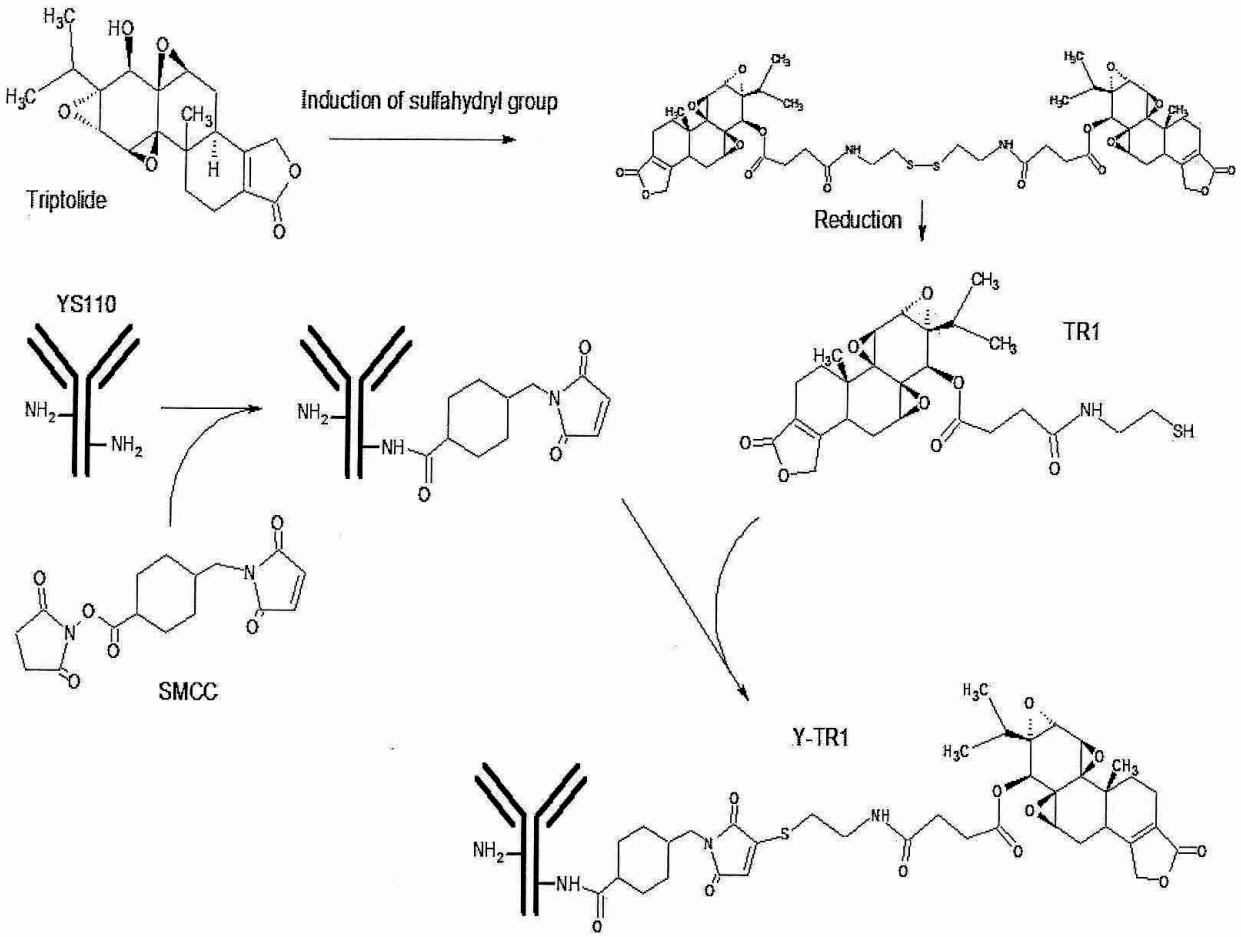

図2　ヒト化抗 CD26 モノクローナル抗体 YS110 への Triptolide 誘導体 TR1 の結合方法

Triptolide に SH 基を導入して，S-S 結合により化学的に安定な二量体とした。S-S 結合は TCEP 還元ゲルを用いて還元され単量体の TR1 としたものを YS110 との結合に用いた。YS110 に heterobifunctional リンカーSMCC を結合させ，さらに TR1 と反応させ，反応産物から非結合 TR1 を除去し，抗体を精製し，Y-TR1 とした。

定発現細胞株（MSTO-wt），CD26 陽性中皮腫細胞 JMN，ヒト T 細胞白血病由来の CD26 陰性 Jurkat 細胞株にヒト CD26 発現ベクターを遺伝子導入した CD26 安定発現細胞株（Jurkat-CD26）と対照としてネオマイシン耐性遺伝子のみを導入した安定発現細胞株（Jurkat-wt）を使用した（順天堂大学・森本幾夫博士より供与）。*In vitro* での抗がん作用は，培養液に Triptolide, TR1, YS110 および Y-TR1 を添加し，48 時間後に WST-1 アッセイで評価した（図 4 および表 1）。その結果，CD26 陽性 JMN 細胞では，Triptolide はきわめて強い増殖抑制効果を示し（half maximal（50%）inhibitory concentration（IC50）：15 nM），Triptolide 誘導体である TR1 は，IC50 でおよそ 1/12 に低下していた（IC50：180 nM）。また MSTO 細胞でも Triptolide では IC50 が 10 nM，TR1 では IC50 が 250 nM と低下していた。この活性の低下は，Triptolide への SH 基の導入によるものと推測された。一方，YS110 に TR1 を結合させた Y-TR1 は，JMN 細胞において，YS110 では 100 μg/mL でプラトーとなり，その時の増殖抑制率は 20%であったが（IC50 測定不能），Y-TR1 では，50 μg/mL でプラトーとなり，その時

表1　培養がん細胞株へのTriptolide, TR1およびY-TR1の増殖抑制効果

細胞株	由来	CD26発現	IC50 of Triptolide (nM)	IC50 of TR1 (nM)	IC50 of Y-TR1 (μg/mL)
MSTO-wt	中皮腫	−	10	250	35
MSTO-CD26 clone12	中皮腫	+	10	250	10
JMN	中皮腫	+	15	180	25
Jurkat-wt	白血病	−	10	ND	100
Jurkat-CD26	白血病	+	6	ND	30

IC50：half maximal（50%）inhibitory concentration

ヒト悪性中皮腫由来MSTO-211H細胞は，CD26発現のない細胞株である。この細胞株にヒトCD26発現ベクターを遺伝子導入し，CD26安定発現細胞株MSTO-CD26 clone 12を作製して使用した。対照として，MSTO-211H細胞にネオマイシン耐性遺伝子のみが導入されたMSTO-wt細胞株を使用した。ヒト悪性中皮腫由来JMN細胞株はCD26の高発現を有する細胞である。またヒトT細胞白血病由来のJurkat細胞株はCD26発現のない細胞株である。この細胞株にヒトCD26発現ベクターを遺伝子導入し，CD26安定発現細胞株Jurkat-CD26と対照としてネオマイシン耐性遺伝子のみが導入された細胞株（Jurkat-wt）を作製して使用した。

その結果，Triptolideに比べてTR1のIC50は，1/12-25へ低下していた。またY-TR1による細胞増殖抑制能について検討したところ，CD26発現細胞はCD26の発現がない細胞と比較して，IC50は1/3.3-3.5と低下していた。

の増殖抑制率は85％を示した（IC50：25 μg/mL）（図4）。またY-TR1の効果において，CD26発現MSTO-CD26細胞とJurkat-CD26細胞では，CD26を発現しないMSTO-wt細胞とJurkat-wt細胞と比べて，IC50は1/3.3-3.5と低下していた（表1）。これらの結果は，TR1はTriptolideに比べて抗がん活性が低下しているものの，YS110との結合によりY-TR1はYS110よりも強い抗がん効果を発揮することが明らかとなった。

次に*in vivo*におけるY-TR1の抗がん効果を検討した。ヒト悪性中皮腫細胞株JMNを免疫不全マウスNOD/SCIDに皮下移植したモデルを用いて，マウス抗ヒトCD26モノクローナル抗体14D10，YS110，Y-TR1の造腫瘍性への影響を解析した。JMN細胞移植後，3日目より，上記の抗体を8 mg/kg体重で週3回腹腔内投与し，3週後に腫瘍を摘出して，腫瘍の重量を計測した。その結果，対照群（ヒトIgG1投与）と比較して，YS110投与群では30％程度の抑制効果を示したが，Y-TR1投与群では，75％までの高い抑制効果を示した（図5）。

6　考察

CD26は，正常ではTリンパ球をはじめとした様々な細胞に発現しており，細胞の接着や増殖などにおいて多彩な機能を有している分子である。またCD26は，C末端にセリンプロテアーゼの一つであるDipeptidyl Peptidase-4（DPP-Ⅳ）を持つが，この阻害剤は糖尿病の治療薬として世界中で広く使用されている。一方，がん細胞において，CD26は広汎ながん腫において高発現がある。ヒト化抗CD26モノクローナル抗体YS110は，このがん細胞表面のCD26に結合

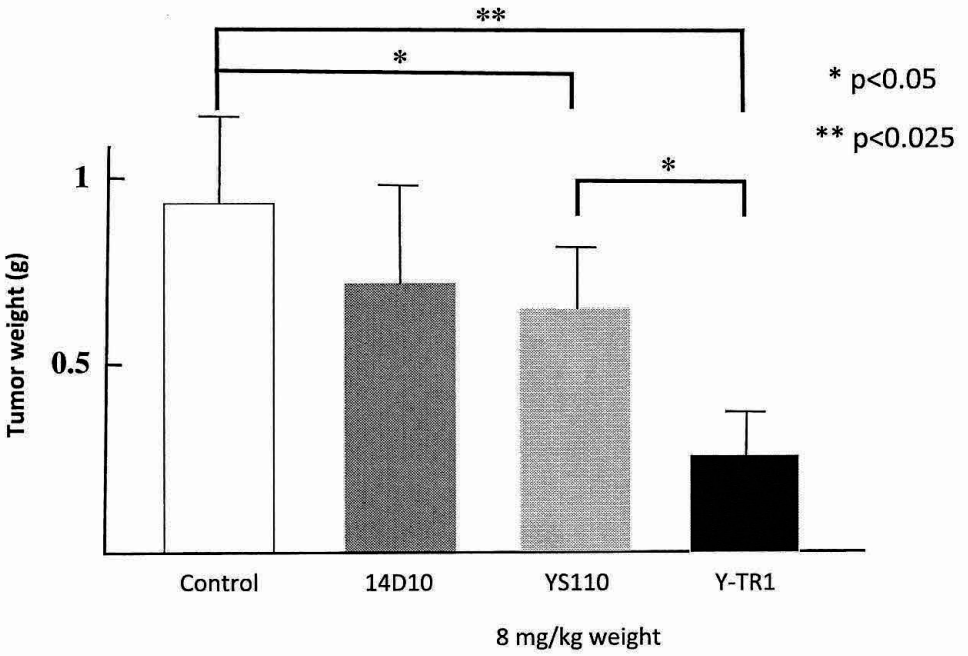

図５　Y-TR1 のヒト中皮腫細胞株に対する *in vivo* での増殖抑制効果

ヒト悪性中皮腫細胞株 JMN を免疫不全マウス NOD/SCID に皮下移植したモデルを用いて，マウス抗ヒト CD26 モノクローナル抗体 14D10，YS110，Y-TR1 の造腫瘍性への影響を解析した。JMN 細胞移植後，3 日目より，上記の抗体を 8 mg/kg 体重で週 3 回腹腔内投与し，3 週後に腫瘍を摘出して，腫瘍の重量を計測した。対照群（ヒト IgG1 投与）と比較して，YS110 投与群では 30％程度の抑制効果を示したが，Y-TR1 投与群では，75％までの抑制効果を示した。

すると CD26 とともにエンドサイトーシスにより細胞質内に取り込まれ，さらに核へ移行する。この YS110 による CD26 の核移行が，正常 T リンパ球や血管内皮細胞では見られないことから，がん細胞特異的な現象と考えられる。このがん細胞特異性から，YS110 が，がん細胞の核への物質輸送に有用と考え，本稿では，核内へ移行した CD26 が，RNA polymerase Ⅱのサブユニット POLR2A 遺伝子の転写を抑制することで細胞増殖抑制を惹起することに着目して，YS110 へ RNA polymerase Ⅱの基本転写因子群である TFⅡH 阻害剤を結合させた ADC の開発について述べた。この YS110 は，さらに毒性が強く全身投与できない様々な抗がん分子や分子標的薬を結合させることで，広大な可能性を有していると考えている。

RNA polymerase Ⅱは，タンパク質をコードする遺伝子のほとんどの転写に必須な酵素であり，細胞の生存・維持・増殖に不可欠な分子である。そのため，以前よりその阻害剤や RNAi が抗がん分子としての可能性が考慮されていた[14]。最近，Liu らは，大腸癌において，p53 遺伝子の近傍に RNA polymerase Ⅱのサブユニット POLR2A 遺伝子があることで，p53 遺伝子の欠失とともに POLR2A 遺伝子も欠失している症例が多いことを見出し，これらの POLR2A 遺伝子のヘテロ欠失がある大腸癌細胞では，毒性が強い RNA polymerase Ⅱ阻害剤アマニチンが，より低濃度で抗がん作用を示すことを報告した[15]。このことから p53 遺伝子の欠失があるがん症例においては，ドラッグデリバリーの方法を駆使すれば，RNA polymerase Ⅱ阻害剤が抗がん剤として使用できる可能性を示唆しており，実際に，Liu らはアマニチンを結合させた抗

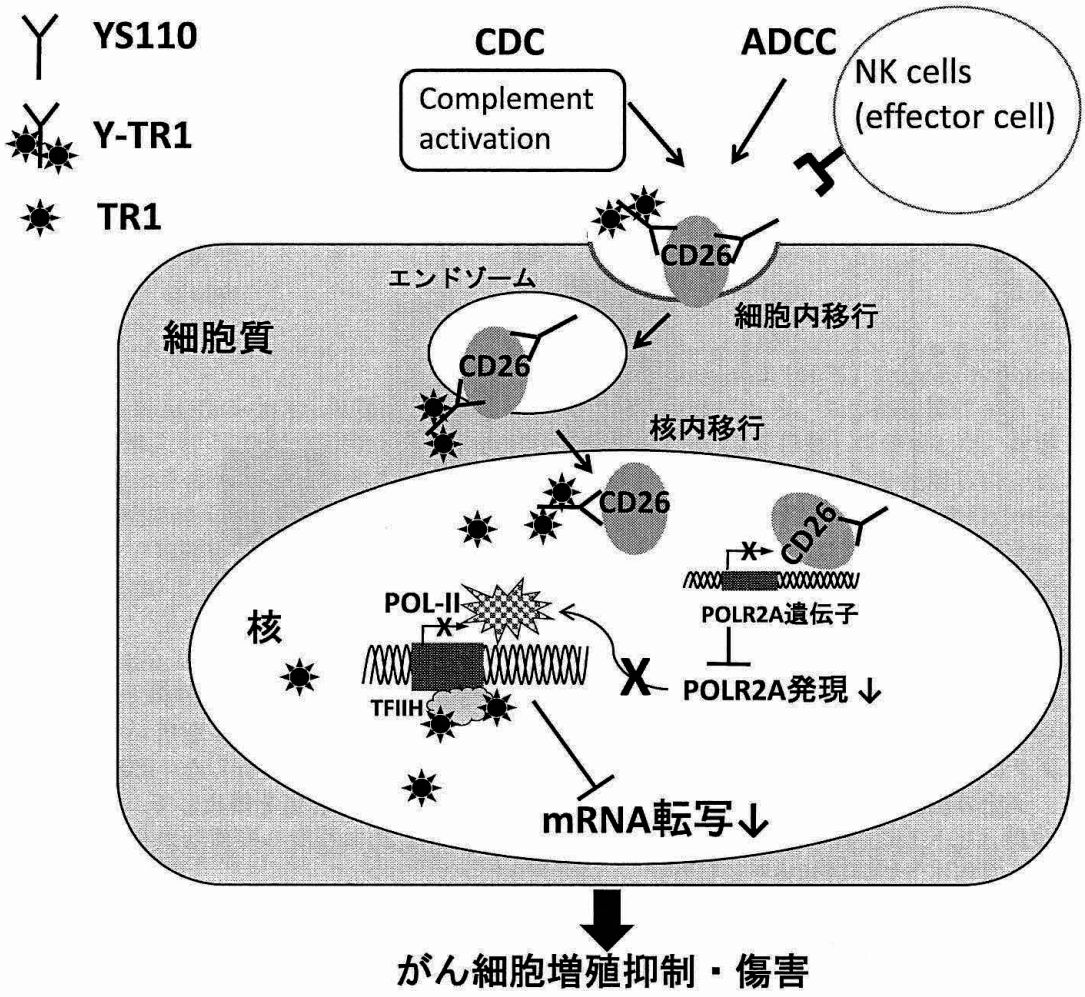

図6　ヒト化抗 CD26 モノクローナル抗体 YS110 への TFⅡH 阻害剤結合による新規 ADC の抗がん機序
YS110 はがん細胞表面 CD26 と結合し，その後，カベオリン依存性に細胞質に移動し，さらに核内に移行することで，RNA polymerase Ⅱのサブユニット POLR2A の転写を抑制する。この YS110 に TFⅡH 阻害剤 Triptolide 誘導体 TR1 を結合させた ADC は，がん細胞の細胞質・核へ TR1 を運搬し，核内で TFⅡH を阻害することにより RNA polymerase Ⅱの機能障害を惹起し，多くの遺伝子の mRNA 転写を阻害することにより，がん細胞の増殖抑制および細胞傷害を誘導する。

epithelial cell adhesion molecule 抗体が，p53 遺伝子の欠失がある大腸癌細胞を移植したマウスモデルで腫瘍を縮小させることに成功している[15]。この点において，本稿で紹介した YS110 は，YS110 による POLR2A 転写抑制による発現低下を誘導することから，p53 遺伝子欠失に伴った POLR2A 遺伝子欠失がない症例においても有効であり，さらに Y-TR1 という新規 ADC が，一つの薬剤で POLR2A 発現低下と THⅡF 阻害の両面から RNA polymerase Ⅱの機能阻害を行うことで，より効果が高く，耐性ができにくい分子標的療法となりうると期待される。

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石綿関連疾患の病理とそのリスクコミュニケーション

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石綿の曝露はヒト体内で何を起こすのか
培養細胞を用いたモデル実験

山田　健人

1　石綿のヒトへの影響－培養細胞を用いた研究の現状

石綿が，肺に石綿肺を，胸膜に胸膜プラークや中皮腫を引き起こす．そこで肺や胸膜から取り出した細胞が石綿に対してどのような反応をするかを調べることは，このような病気の発症メカニズムや予防，治療を考える上で重要である．これまでに試験管内で培養される様々な細胞に石綿を加えて起こる現象や細胞内での生化学的，分子生物学的知見が集まってきている．しかし，試験管の中で石綿が培養細胞をただちにがん化することはないため，試験管内で観察される一つ一つの現象・知見を組み合わせることで，体内ではおそらくこのようなことが積み重なって長い年月を経て細胞のがん化が起こるのだろう，と推測することになる．

石綿に暴露している作業者は，肺胞腔内に好中球（白血球の一つ）の浸潤が多いことが知られている[1]．そこでヒトⅡ型肺胞上皮細胞株 A549 およびヒト気管支上皮細胞を石綿で刺激したところ，培養液中にインターロイキン 8（IL-8）という好中球を呼びよせて刺激するサイトカインの分泌を亢進させた[2]．しかし石綿以外の酸化チタンなどの物質では，IL-8 の誘導はみられなかった．また同様に石綿でラット肺上皮細胞および中皮細胞を 20 分間刺激したところ，4～8 時間後に炎症や線維化に関連する転写因子 NF κ B の一部 p65 が誘導され，核内への移行が進み DNA への結合能が高まった[3]．さらに石綿をラットに経気道的に吸引（6 時間／日，5 日／週，5 または 20 日）させたところ，肺における p65 の誘導は，5 日目から認められ，20 日間持続した．このことは石綿による肺や中皮細胞の刺激で惹起される炎症やその後の線維化の進行に必要な期間，炎症を引き起こしうることを示した[3]．またマウス腹腔マクロファージ（白血球のひとつ）を石綿で刺激すると，マクロファージは石綿を貪食するとともに，活性酸素の産生が上昇し，マクロファージは細胞死に陥る[4]．この時に，あらかじめ石綿から鉄イオンを除去しておくと，このマクロファージの細胞死はみられなかった．このことから石綿がマクロファージに貪食されるときに鉄イオンがあると活性酸素の産生が亢進して細胞死を引き起こすと推測される．一方，石綿の刺激は，マウス胸腔中皮細胞の中のグルタチオン（3 つのアミノ酸からなる抗酸化分子で活性酸素から細胞を保護する）を減少させることで，石綿刺激後にがん遺伝子である *c-fos* および *c-jun* を誘導する[5]．この現象は，石綿が細胞内において活性酸素から保護するレドックス（細胞内の酸化・還元状態）を変化させることで，がん化の最初の一歩を踏み出させている可能性を示唆する．正常ヒト中皮細胞を石綿とともに培養すると石綿の濃度依存性に細胞死が誘導される．しかし一定の濃度で石綿処理を行うと正常ヒト中皮細胞は腫瘍壊死因子 TNF- α（サイトカインの一種でがん細胞を殺傷することで発見された）を産生するとともに TNF- α 受容体が誘導される．このように一定量の TNF- α が誘導されると TNF- α 受容体を介して NF κ B が活性化して，その結果として正常ヒト中皮細胞は石綿に対して抵抗性を獲得する．この時，石綿と TNF- α の両方の存在下で正常ヒト中皮細胞を培養していると，頻度は低いながら通常の培養では観察できないような異常な細胞分裂像を認めるようになる[6]．これらの結果は，石綿で傷つけられながらも生き残った中皮細胞は，サイトカインの誘導

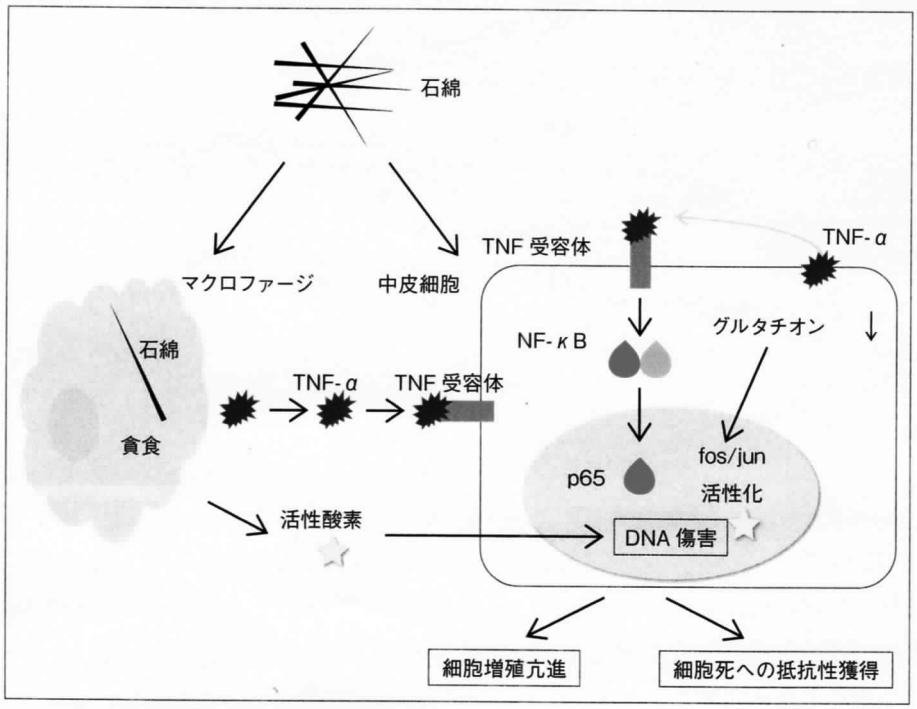

図 1　石綿のヒト培養細胞への影響

を介してさらなる細胞傷害や細胞死に抵抗性を獲得して，長い年月を経る間に悪性化していくことを推測させる．この石綿による中皮細胞の細胞死においては，細胞死の実行分子の一つである poly（ADP-ribose）polymerase の活性化と high mobility group box 1（HMGB1）タンパク質の核から細胞質への移動と細胞外への分泌が観察された[7]．HMGB1 の細胞外への分泌は，マクロファージからの TNF-α 産生を亢進させ，上記のように中皮腫細胞での石綿に対する抵抗性獲得を誘導した．実際に，血清中の HMGB1 濃度は，石綿暴露作業者において有意に高値を示した（**図 1**）．

このように石綿で培養細胞を刺激する試験管内の実験からは，石綿肺や中皮腫などの石綿関連疾患の発症メカニズムに関する多くの基礎的な知見が得られる．この知見をもとに以下に述べるモデル動物を使った研究や実際に石綿関連疾患患者の病態の解析に進み，これらの知見が本当に石綿関連疾患に関与しているかを判明していくことが重要と思われる．

2　中皮腫の培養細胞株

1）培養がん細胞

培養細胞は，最低限の必須な栄養を含む液体（培養液）にウシ血清を加えて，プラスチック製の培養皿の中で無菌的に育てられる．培養皿は，一定の濃度の二酸化炭素ガスの中で一定温度の培養器の中に置かれ，2～3 日に一度，培養液を取り替えることで細胞を増殖させる．細胞が多くなってきたら，細胞を培養皿から剥がすために，酵素液を使い，剥がした細胞の一部を次の培養皿に移して，再び培養することで細胞を増殖させる（継代培養）．ヒト成人の正常な細胞は，通常は一定期間，培養すると死滅してしまう．がん組織から採取されたがん細胞も，大部分の細胞は死滅するが，一部の細胞は培養液に順応して増殖を始める．増殖してきたがん細胞は，継代培養を繰り返せるようになり，冷凍保存が可能となる．このように安定して増殖するとともに凍結保存ができる細胞は細胞株とよばれる．

がん組織は様々な状態（染色体の質・量あるいは特定の遺伝子変異の有無，生化学的状態など）の細胞が集合しているものであることから，細胞株として均質なものを得るために，がん細胞を一つだけ取り出して，その単一の細胞から増殖させることで，クローン（または亜株）を得ることができる．このクローンは，単一細胞由来なので，染色体や遺伝子の状態や栄養要求などの生化学的状態が一定と推測され，より厳密ながん細胞株となる．このように，がん細胞株は，患者のがん組織に由来し，一定の組成の液体の中で増殖性を有し凍結保存に耐えるものである．さらにクローン化した細胞株では培養条件

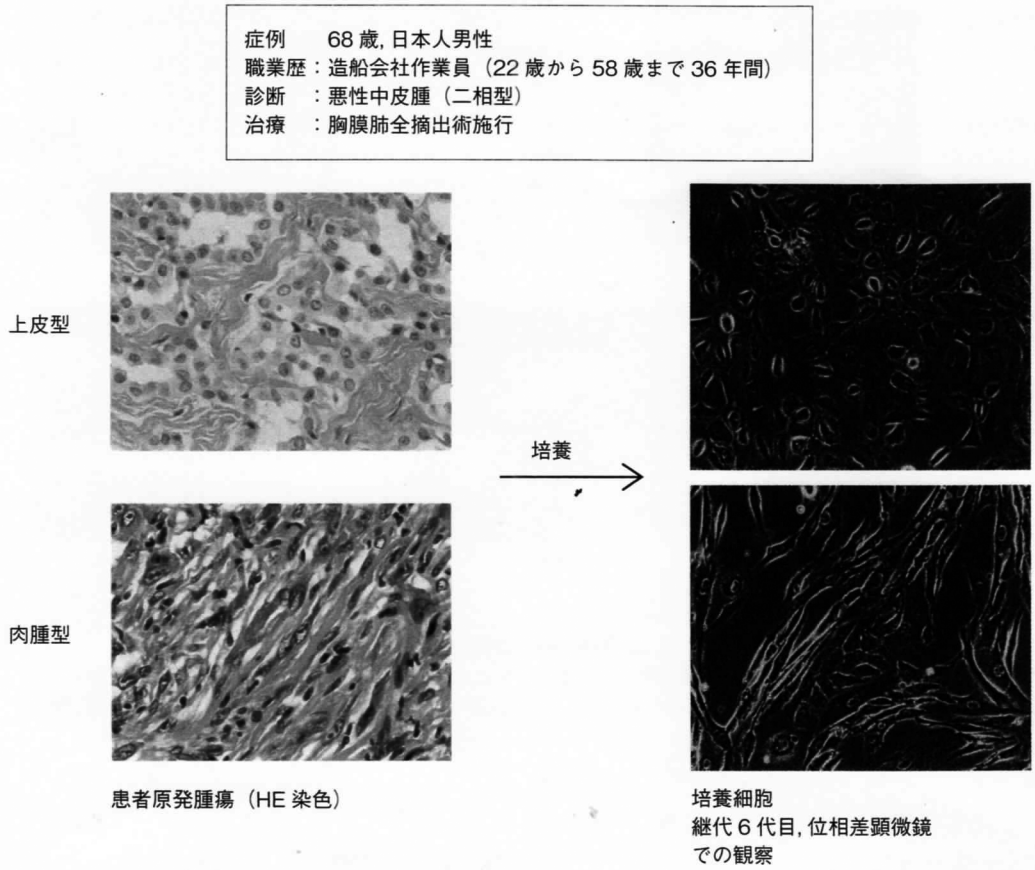

図2　中皮腫からの細胞培養
上皮型（HE染色，対物×20）と肉腫型（HE染色，対物×20）からでは培養細胞の形が異なる．
上皮型からは類円形の細胞，肉腫型からは紡錘形の細胞が増殖してくる．

を同じにすれば世界中のどこでも同様の培養が可能であり，大量の細胞が均一に安定して得られることから，さまざまながん研究に使用できる貴重で便利なものである．

2）中皮腫の培養細胞

中皮腫はきわめて悪性度の高いがんであるが，腫瘍としての進展速度は比較的遅いと考えられている．患者の同意のもとに中皮腫組織を採取して，そこからがん細胞を培養すると，患者の体腔内と同様に培養皿に接着して皿底に広がりながら増殖する．また胸水に浮遊していた中皮腫細胞を培養しても，同様に培養皿に接着して増殖する．患者の中皮腫組織や胸水から，いろいろな培養方法で多くの細胞株がこれまで樹立されてきた[8～11]．中皮腫は，患者の胸腔，腹腔，心嚢腔などの体腔を覆う中皮細胞から発生する腫瘍であり，別項目2で詳細に述べられているように体腔を這うように進展し，もともとあった組織に浸潤していく腫瘍である．

中皮腫は，その組織を顕微鏡で観察すると上皮型，肉腫型とこれらの両者が混在した二相型に分類される．これまで樹立されてきた中皮腫細胞株もこれらの様々な型の腫瘍から由来していて，培養皿の底で増殖する細胞の形は，由来となる型と相関することが多く，たとえば上皮型中皮腫由来の細胞株は，目玉焼きのような円形であったり，下駄のような長方形であったりして，それぞれが畳を並べたように互いの細胞が肩を寄せ合いながら増殖することが多い（**図2**）．

一方，肉腫型は紡錘形の細胞からなることが多く，いろいろな方向を向きながら互いに重なり合いながら秩序に乏しく増殖する（**図2**）．二相型からも細胞株が樹立されているが，前述したように多くの細胞株はクローン化されているため，上皮型か肉腫型かどちらかに類似しているもののみが得られている（両方の型が混じっている細胞株は樹立が難しい）[8, 9]．たとえば，JMN細胞は1982年に樹立し報告された細胞株で，腹膜原発の二相型中皮腫の患

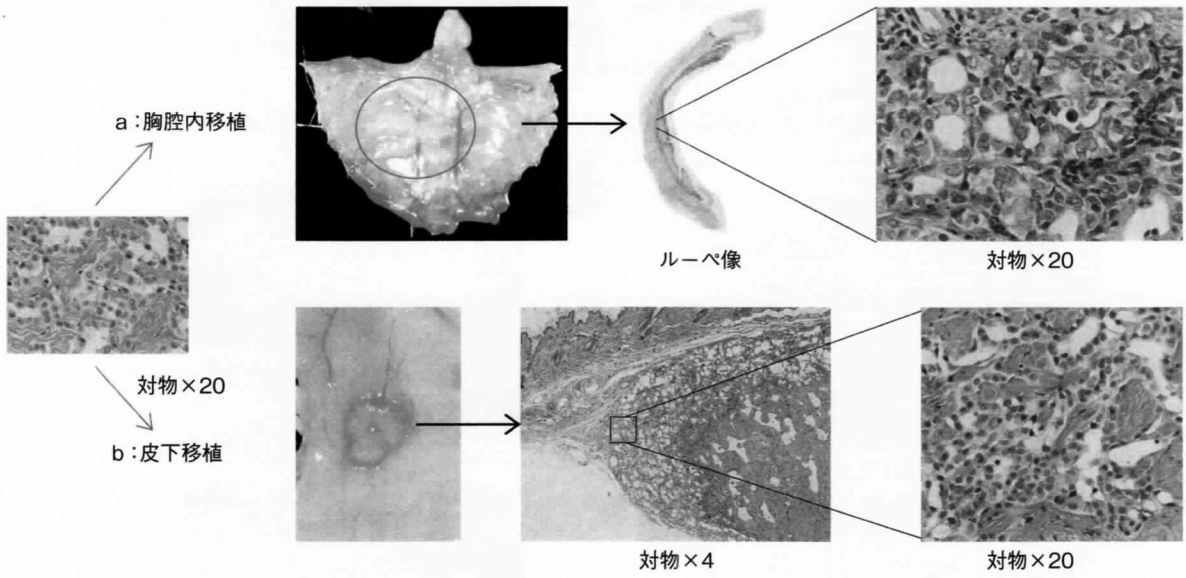

図3　中皮腫手術検体を用いた中皮腫動物モデル
患者（**図2**）の中皮腫組織（上皮型）をマウスへ同所移植（**a**：胸腔内移植）と異所移植（**b**：皮下移植）し，3カ月後にマウス胸腔（**a**）と皮下組織（**b**）に形成された腫瘍（いずれも上皮型）が観察される．胸腔内では，広がりをもった腫瘍が形成されるが（赤丸），皮下組織では，限局した腫瘤の形成にとどまる．染色はいずれもHE染色．

者の腹水から得られたものであり，後述するように新しい動物モデルや治療法の開発に役立っている貴重な細胞である[8]．この報告では，細胞株は培養において紡錘形細胞からなり，継代を重ねると紡錘形細胞はさらに細長くなり，重なり合いが強くなった．また，この細胞を免疫不全マウスの皮下に移植して形成された腫瘍では，上皮型や肉腫型の両方ができたと記載されている．ところが，現在，2015年の時点で入手できるJMN細胞は，紡錘形細胞からなり，免疫不全マウスの皮下や胸腔あるいは腹腔に移植すると肉腫型中皮腫のみが形成され，上皮型は全く認められない．このことは，当初は上皮型の細胞が混在していたものの，継代を続けるうちに，培養状態による選別がかかり，その条件で増殖しやすいものが優勢に増殖していくことで，細胞に様々な変化が加わってきたものと推測される．このように培養細胞株を用いたがん研究においては，最初に樹立されて報告された時とは細胞の性格やふるまい（染色体や遺伝子の構造・状態変化）が異なっている可能性があることを注意し，今，使用している細胞株がどのような状態であるかを確認する必要があることを示している．

3　中皮腫モデル動物の作出

1）同所移植と異所移植

ヒトがん組織を免疫不全マウスに移植することで，ヒトがん組織・細胞を生体内で観察することが可能である．通常，ヒトがんは，外表から観察しやすいマウス皮下組織に移植し，その後，時間とともに大きくなる腫瘍のサイズを計測する．このモデルを使うと，そのがんの増殖や細胞死に関わる遺伝子や機能分子の解析が可能であり，また新しい抗がん療法の効果を調べることも可能である．しかし，マウス皮下組織にヒトがんがある状態は，患者でいえば皮膚に転移した腫瘍をみていることになるが，がんが発生する場所にヒトがんを移植することで，原発腫瘍と似た状態でヒトがんを観察することが可能となる．たとえば，膵がんであればマウス膵臓に，肝がんであればマウス肝臓に移植する訳である．このような移植を同所移植と呼ぶ．

一方，ヒト膵がんや肝がんをマウス皮下組織に移植することを異所移植と呼ぶ．さて中皮腫の場合は，胃がん，肺がんや乳がんなどの一般的ながんとは，発生部位や組織像が異なるだけでなく，その増殖様式が大きく異なる．中皮腫では腫瘍が体腔表面を這うように進展し，さらに周囲の組織に浸潤することで，胸腔であれば胸壁や肺表面での強い痛みを引き

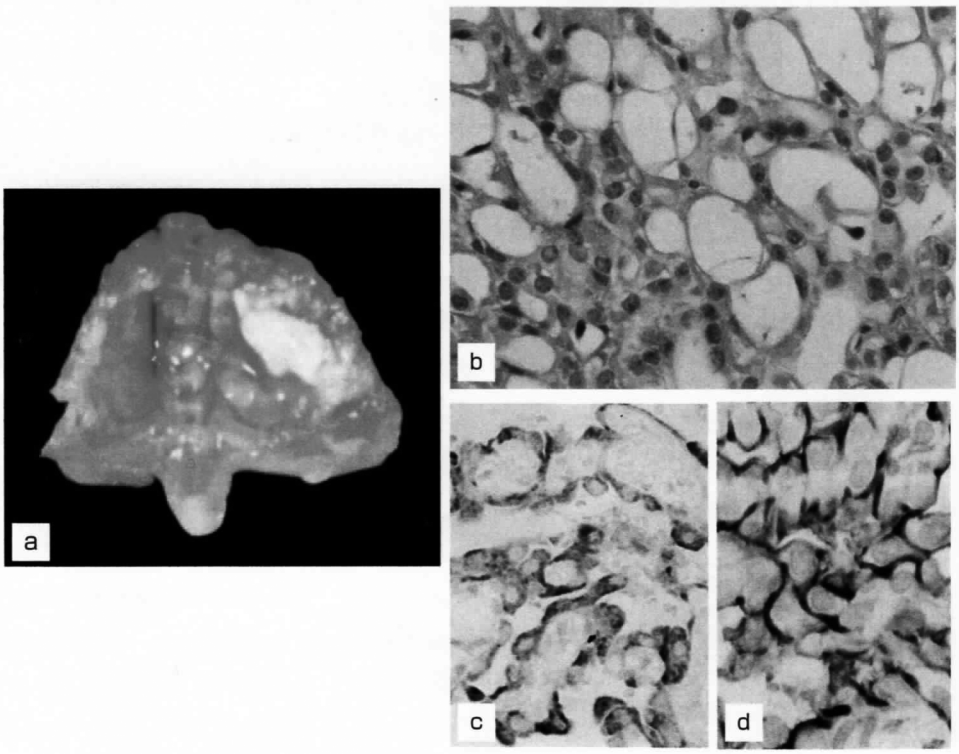

図4　図2の腫瘍から樹立したKMES細胞株の同所移植による中皮腫モデル
a：マウス胸腔を裏側から観察した．白色から薄いピンク色の腫瘍が胸腔を這うように進展している．
b：上皮型中皮腫の組織像が再現されている（HE染色，対物×20）．
c：Calretinin染色陽性像（褐色．対物×20）．
d：D2-40染色陽性像（褐色．対物×20）．

起こし，さらに進行すると呼吸機能低下を惹起する．このような特徴を持つ中皮腫を研究するには，なるべく患者の状態と近いモデル動物を作ることが重要である[12]．そこで中皮腫患者の同意を得て手術で摘出された腫瘍から小片組織を採取して，免疫不全マウスの胸腔内（同所）と皮下組織（異所）に移植した．移植後3カ月の時点でマウスを解剖して，胸腔内と皮下組織を観察した（**図3**）．このように胸腔内に同所移植すると腫瘍は一カ所に留まらず，広がって増殖する傾向が認められ，患者の病態に近い像を示す．一方，皮下組織に異所移植した場合には腫瘍は一カ所に腫瘤を形成する．このように同じ腫瘍組織でも移植場所によって進展様式が異なることから，なるべく患者の病態に近いモデルを作って行くことが大事と考える．

2）腫瘍組織の移植と培養細胞株の移植

前述したように患者の中皮腫組織をマウスに移植して疾患モデルを作ることは可能であるが，手術をして組織を採取したときにのみ可能なモデルであり，患者によって，あるいは腫瘍ごとに様々な病態がマウスに再現されると考えられる．また，この場合には**図2**のような腫瘍を形成するのに3～6カ月かかるのが通常である．そこで，どこの研究室でも均一な再現性が高いモデルが素早く可能にするのが，中皮腫培養細胞株を用いたモデル動物である．培養細胞株の5万個～50万個の細胞を免疫不全マウスの胸腔内，腹腔内あるいは皮下組織に移植すると，おおむね3～6週間で腫瘍が形成される．培養細胞においても細胞株を選別することで，胸腔内への同所移植により再現性高く胸腔に沿って広がる進展方式を伴う中皮腫モデルができる[10, 13, 14]．

二相型中皮腫由来の細胞株であるKMES細胞10万個をマウス右胸腔内に移植して4週後にマウスを解剖した（**図4**）．胸壁を心臓，肺側からみると胸腔内に中皮腫腫瘍組織が広範囲にわたって進展増殖していることがわかる（**図4a**）．この進展は移植した右胸腔から胸骨の裏側を進み，移植6週後には左胸腔へ達して両側胸腔内に血性胸水を大量に貯留させる．またこの腫瘍について顕微鏡的観察を行うと

患者の腫瘍組織で観察された上皮型の成分の中皮腫ときわめて類似した組織像を示すことがわかった（**図 4b**）．さらにこの腫瘍組織について，中皮腫のマーカー（別項「中皮腫診断での体腔液細胞診の特徴と考え方」103 頁参照）である calretinin と D2-40 について免疫染色を行うと両者ともに陽性であることが明らかとなった（**図 4c, d**）．このように培養細胞株を移植することで中皮腫動物モデルを作出することで，世界中の研究室で均質で安定した再現性高い疾患モデルが利用できることことから，多くの培養細胞株がそれぞれの特徴を生かした分子レベルでの中皮腫の研究や新しい治療法の探索に広く利用されている．

4 培養細胞株を用いた中皮腫モデルあるいはモデル動物を用いた診断・治療法の開発

1）培養細胞株を用いた中皮腫の研究と治療法の開発

中皮腫細胞株を用いることで中皮腫の増殖や細胞死の分子機構を解析できることから，多くの研究がなされている．しかし，中皮腫は腫瘍組織も多彩で不均一であり個人差も大きい上，細胞株は樹立する過程でさまざまな要因から選別されることで，もとの腫瘍の一部の細胞（培養に順応できて，増殖能が高いもの）のみが得られると推測される．さらに一つの研究で用いられる細胞株は，数種類に限られてしまうことから，中皮腫全般における基盤となる分子機構を明らかにしていくには困難が多い．

6 種類の中皮腫細胞株（上皮型 1 株，肉腫型 1 株，二相型 4 株）を用いて，抗がん剤である selenite，bortezomib，carboplatin，pemetrexed，doxorubicin と gemcitabine とその様々な組み合わせによる増殖抑制効果を比較した報告がある．その中で，selenite 単独では，4 種類の細胞株（上皮型 1 株，二相型 3 株）では増殖抑制が観察されたが，他の 2 種類の細胞株（肉腫型 1 株，二相型 1 株）では効果がみられなかった．また他の単剤あるいは 2 種類の併用では，全体的に上皮型の方が，肉腫型よりも抗がん剤への感受性が高い傾向が認められた[15]．このように培養細胞株を用いて中皮腫のがんとしての性質を明らかにし，抗がん剤の開発を行うためには，上皮型，肉腫型，二相型の多種類の細胞株を併用して用いて行うことが望ましく，細胞株で得られた知見は，必ず患者の中皮腫組織において，本当にその知見が正しいかどうかを慎重に確かめて行くことが重要である．

二相型中皮腫由来 MSTO-211H 細胞株を用いることで，タンパク質の網羅的解析から中皮腫に多く発現している AHNAK 分子が同定された[16]．この分子は，他の中皮腫細胞株 7 種類すべてにおいて高い発現が確認されたが，正常ヒト中皮細胞では発現がみられなかった．さらに患者の中皮腫組織で調べたところ，上皮型，肉腫型および二相型のいずれの組織型においても発現がみられた．次に試験管の中で細胞が運動するときや細胞外マトリックスに浸潤するときにこの AHNAK 分子が積極的に機能していることが，これらの培養細胞株を用いることで明らかとなった．この研究は，中皮腫細胞株に特異的に多く発現している分子をみつけることで，新しいバイオマーカーの発見を行ったものであり，培養細胞を利用することで，その見い出された分子ががん細胞のどのような機能に関係するか，試験管内で探索することを可能にすることを示している．

ここでは多くの例は示せないが，最近，がん研究の中で発見されたマイクロ RNA（タンパク質にはならない非コード RNA）が中皮細胞において新しく見い出されたり[17]，中皮腫において CD26 という細胞膜表面分子が高い発現を示し，細胞接着や浸潤に関与していること，さらに抗 CD26 ヒト化抗体が中皮腫細胞株に対して抗がん効果を示すこと[18]，あるいは細胞内でタンパク質を分解する巨大な酵素複合体であるプロテアゾームの阻害剤が中皮腫細胞株の増殖を阻止すること[19]など，細胞株を利用した中皮腫の研究が蓄積しつつある．

2）モデル動物を用いた中皮腫の研究と診断・治療法の開発

試験管内で培養細胞を使って研究を行うことは，比較的均一な条件の中で生物学的現象を明らかにすることができることから，前述したような機能分子の同定や中皮腫で多く発現している分子の探索にとても有用である．さらに細胞増殖，接着，運動などの細胞の機能を定量的に解析できるのも培養実験の利点である．こうして見い出された分子が，細胞の機能に関わっていることをさらにヒト病態に近い状

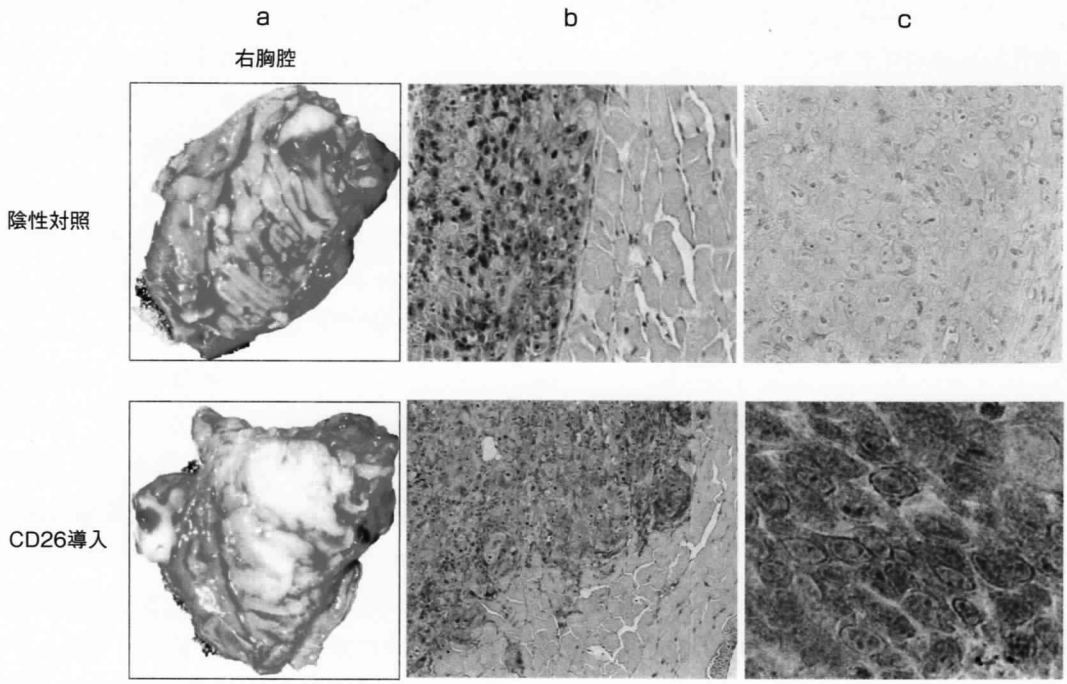

図5　MSTO-211H細胞による中皮腫モデルを用いたCD-26の機能解析

a：CD26発現がない細胞を移植した場合（陰性対照，**上図**）と比較してCD26発現がある場合（**下図**）では腫瘍の増殖が亢進する．

b：胸壁の標本では，陰性対照（**上図**：HE染色，対物×10）では筋組織との境界が明瞭であるのに対して，CD26導入群（**下図**：HE染色，対物×10）では筋組織への浸潤がみられる．

c：CD26発現（褐色）を示す（**上図**：CD26免疫染色，対物×20，**下図**：CD26免疫染色，対物×20）．

態で観察するために，前述したヒト中皮腫モデル動物が有用となる．中でも培養細胞株を免疫不全マウスへ移植して作出するモデルは，がん細胞とがんの環境を形成する周囲の細胞（血管，間質細胞や免疫担当細胞など）と細胞外マトリックス（細胞と細胞の間に存在して，足場や栄養などを提供する）の相互作用を観察することができる．

まずは中皮腫の組織型（上皮型，肉腫型）のモデルについて紹介する．上皮型細胞株STAV-ABと肉腫型細胞株STAV-FCSを免疫不全マウス皮下組織あるいは腹腔内に移植し，8週後にその腫瘍が解析された．その結果，いずれの細胞株も腫瘍の組織型としては上皮型の像を示したが，肉腫型の方が増殖速度が早かった．しかし，第3番染色体のCGHアレイ解析（comparative genomic hybridization，2色の蛍光色素でラベルした検体を競合的にマイクロアレイ上でハイブリダイズさせてコピー数を比較定量する解析技術）からは，培養細胞ではSTAV-AB細胞とSTAV-FCS細胞では，異なるパターンを示したのに対して，マウスに移植した腫瘍では，さらに異なったCGHパターンを示すとともに，2つの細胞株での差異は消失していた[20]．この研究は，培養細胞とモデル動物での細胞を取り巻く環境が異なることが原因で，細胞が増殖とともに腫瘍を形成する過程で，選別される可能性を示唆している．

次に培養細胞株では観察できない研究として，腫瘍とともに増生する血管についての研究を紹介したい．二相型中皮腫患者さんの血性胸水からEHMES-10細胞株を樹立された[10]．この細胞を免疫不全マウスの胸腔内に同所移植すると，5週後には血性胸水の貯留とともに胸腔内に腫瘍が広がっていた．腫瘍を顕微鏡で観察すると，腫瘍の中にはいわゆる腫瘍血管（マウス由来）が多く認められた．そこでEHMES-10細胞における血管内皮細胞の増殖因子であるvascular endothelial growth factor（VEGF）とその受容体VEGFR-2の発現を調べてみると，VEGFとVEGFR-2の両方の発現が高いことが判明した．このことは，この中皮腫細胞では，VEGFがオートクライン機構（みずから産生する増殖因子でみずからを増殖させること）で増殖亢進に寄与していることを示す．その後，同じ研究グループは，このVEGFの中和抗体bevacizumab（VEGFの作用を阻害する抗体）がpemetrexedとの併用により中皮腫の抗がん作用を発揮することを報告している[21]．

Imatinibは，受容体型チロシンキナーゼの阻害剤であり，慢性骨髄性白血病の分子標的薬として開発された分子である．阻害できる受容体型チロシンキナーゼとしては，Bcr-Abl，v-abl，c-abl，PDGF受容体およびc-Kitが知られている．このPDGF受容体が中皮腫において発現が多いことから，PDGF受容体陽性のREN細胞株を用いることで，imatinibがgemcitabineとの併用により抗腫瘍効果を示すことが報告された[22]．この研究でREN細胞株には，ルシフェラーゼ（ホタルが発光するための酵素，レポーター遺伝子とよぶ）の発現ベクターを導入しておき，そのルシフェラーゼ陽性REN細胞を免疫不全マウス腹腔に移植後，4週間経過観察する．マウス体内の腫瘍の様子は，あらかじめルシフェリン（ルシフェラーゼの基質でありルシフェリンがATP存在下にアデニレート化されて発光する）を注射してから，それにより発光した腫瘍をマウス体外から検出できる特別な装置を使って観察する．これにより，マウスを生かしたままで，経時的に腫瘍の増大や縮小を観察することができる（生体バイオイメージング）．この実験により，imatinibは単独では抗がん作用は明らかでないが，gemcitabineとの併用によりマウス体内の腫瘍増殖を抑制すること，そのときに中皮腫組織での細胞分裂は抑えられ，細胞死は増加することが判明した．さらにREN細胞株を移植したマウスをそのまま飼育していくと，gemcitabine単独投与と比較してimatinibとgemcitabineの併用投与により，マウスの生存期間は約20日延長した．この研究は，現在，他の疾患で有用な分子標的薬などの抗がん剤においても，中皮腫における同様の分子機構が解明されれば，中皮腫にも応用が可能であることを示唆している．さらにバイオイメージング技術の発達により，ルシフェラーゼなどのレポーター遺伝子を導入可能な培養細胞株をマウスに移植することで，生きたままで腫瘍の動態が観察できるようになり，がんモデル動物研究はさらに発展することが期待される．

5　中皮腫におけるCD26の機能とヒト化抗体を用いた分子標的療法

最後に中皮腫におけるCD26の機能とヒト化抗体を用いた分子標的療法について紹介する．中皮腫症例においては，上皮型の88％にCD26が高発現しているが，肉腫型での発現頻度は低い[23]．このCD26は，Tリンパ球の一部や血管内皮細胞，腎臓近位尿細管上皮などで発現が知られているが，がんにおける機能は不明であった．そこでまず中皮腫におけるCD26の機能を解析したところ，JMN細胞株を用いた検討から，CD26がファイブロネクチンやⅠ型コラーゲンとの接着に関与すること，抗CD26ヒト化モノクローナル抗体により細胞増殖が抑制されることが明らかとなった[18]．このCD26を発現ベクターにより，CD26陰性中皮腫細胞株MSTO-211Hに遺伝子導入して，CD26陽性MSTO-211H細胞を作製し，免疫不全マウス胸腔内に同所移植した．その結果，CD26陽性MSTO-211H移植マウスでは，CD26陰性細胞移植群と比較して胸腔内腫瘍の増大が促進され，移植6週後において腫瘍はより大きく厚くなっていた（**図5a，c**）．さらに腫瘍を顕微鏡的に観察すると，CD26陰性細胞移植腫瘍では，マウス胸壁筋組織への浸潤は示さず，境界が明瞭であったのに対して，CD26陽性細胞移植腫瘍では，胸壁筋組織に浸潤性の増殖を示した（**図5b**）．このことは，培養細胞を用いた細胞運動，浸潤の研究では限界があった中皮腫の特徴（胸腔に広がりながら胸壁や肺に浸潤性増殖する）をこのモデルでは再現しうることを示す．CD26陽性MSTO-211H細胞を同所移植するモデルにおいて，抗CD26ヒト化モノクローナル抗体を定期的に腹腔内投与すると，CD26陰性細胞移植群では腫瘍抑制効果はないが，CD26陽性細胞移植群では腫瘍径が1/2以下，腫瘍重量では1/3以下となった[14]．

このようにヒト中皮腫細胞株を用いることで，新たな分子を探索し着目して試験管内で分子・細胞レベルの詳細な研究を行い，さらに細胞株をマウスへ移植することでより疾患に類似した病態を再現するモデル動物を作って，そこであらためて分子の生体内での機能を解析できる．ときにはその分子を標的にした新規の治療法を考案して，その効果や安全性を動物モデルで評価する．このように培養細胞と実験動物を行ったり来たりしながら，中皮腫の研究を進めていくことが新しい診断法や治療法に繋がるものと期待している．最後に，これらの細胞培養株は患者の勇気ある決意と寛容な心に基づいて腫瘍組織

を提供していただいたことで初めて存在するものであり，すでに樹立して40年以上経っているもの，最近，樹立されたものを含めて感謝の念を述べたい．

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A novel role for CD26/dipeptidyl peptidase IV as a therapeutic target

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1. ABSTRACT

CD26 is a 110 kDa surface glycoprotein with intrinsic dipeptidyl peptidase IV activity that is expressed on numerous cell types and has a multitude of biological functions. The role of CD26 in immune regulation has been extensively characterized, with recent findings elucidating its linkage with signaling pathways and structures involved in T-lymphocyte activation as well as antigen presenting cell-T-cell interaction. In this paper, we will review emerging data on CD26-mediated immune regulation suggesting that CD26 may be an appropriate therapeutic target for the treatment of selected immune disorders as well as Middle East respiratory syndrome coronavirus. Moreover, we have had a long-standing interest in the role of CD26 in cancer biology and its suitability as a novel therapeutic target in selected neoplasms. We reported robust *in vivo* data on the anti-tumor activity of anti-CD26 monoclonal antibody in mouse xenograft models. We herein review significant novel findings and the early clinical development of a CD26-targeted therapy in selected immune disorders and cancers, advances that can lead to a more hopeful future for patients with these intractable diseases.

2. INTRODUCTION

CD26 is a 110-kDa cell surface glycoprotein with known dipeptidyl peptidase IV (DPPIV, EC3.4.1.4.5) activity in its extracellular domain (1-3), capable of cleaving amino-terminal dipeptides with either L-proline or L-alanine at the penultimate position (3). CD26 activity is dependent on the expressing cell type and the microenvironment which influence its multiple biological roles (4-7). CD26 plays an important role in immunology, autoimmunity, diabetes and cancer (8-12). Interacting directly with various other cell surface and intracellular molecules, CD26 can regulate receptor specificity and the function of various interleukins (ILs), cytokines and chemokines via its DPPIV activity (13).

In this review, we summarize our recent work on CD26/DPPIV that elucidated its suitability as a potential therapeutic target in selected immune diseases and cancers. We also discuss our current knowledge of the molecular mechanisms of CD26/DPPIV-mediated T-cell regulation, focusing particularly on CD26/DPPIV role in immune checkpoint pathways and programs associated with human immune regulation. In addition, we describe CD26/DPPIV involvement in cancer immunology.

3. IMMUNE MEDIATED DISORDERS

3.1. Chronic graft-versus-host disease

3.1.1. T cell costimulation in chronic graft-versus-host disease

Graft-versus-host disease (GVHD) is a severe complication and major cause of morbidity and mortality following allogeneic hematopoietic stem cell transplantation (alloHSCT) (14). Based on differences in clinical manifestations and

histopathology, GVHD can be divided into acute and chronic forms (14). Acute GVHD (aGVHD) and chronic GVHD (cGVHD) are traditionally diagnosed primarily by time of onset, with cGVHD occurring after day 100 of transplantation (15). However, cGVHD has distinct clinicopathologic features and is often diagnosed based on these features regardless of time of onset, being characterized by cutaneous fibrosis, involvement of exocrine glands, hepatic disease, and obliterative bronchiolitis (OB) (16, 17). OB, characterized by airway blockade, peribronchiolar and perivascular lympho-fibroproliferation and obliteration of bronchioles, is a late-stage complication of cGVHD (18). Patients diagnosed with OB have a 5-year survival rate of only 10 to 40%, compared to more than 80% of patients without OB (19, 20). Furthermore, while multiple strategies to control cGVHD involving T cell depletion from the graft or global immunosuppression have been developed, cGVHD is still a common clinical outcome in many alloHSCT patients (17, 21). In addition, immunosuppression potentially abrogates the graft-versus-leukemia (GVL) effect, associated with increased relapses following alloHSCT (22). Novel therapeutic approaches are thus needed to prevent cGVHD without eliminating the GVL effect.

GVHD is initiated when donor-derived T cells are primed by professional antigen presenting cells (APCs) to undergo clonal expansion and maturation (14). Costimulatory pathways are required to induce T cell proliferation, cytokine secretion and effector function following antigen-mediated T cell receptor activation (23), and the important role of costimulatory pathways in transplant biology has been established (24). CD26 is associated with T cell signal transduction processes as a costimulatory molecule, as well as being a marker of T cell activation (1, 25, 26). We previously showed that CD26-mediated costimulation in human CD4 T cells exerts an effect on production of T_H1 type proinflammatory cytokines such as interferon (IFN)-γ (6). Moreover, $CD26^{high}$CD4 T cells respond maximally to recall antigens with a high competence for trafficking to inflammatory tissues and for antibody synthesis by B cells (6, 26). We also showed that CD26-caveolin-1 interaction leads to activation of both CD4 T cells and APCs (27-29). More recently, we demonstrated in *in vitro* experiments that blockade of CD26-mediated T cell costimulation by soluble Fc fusion proteins containing the N-terminal domain of caveolin-1 (Cav-Ig) diminished primary and secondary proliferative responses not only to recall antigen, but also to unrelated allogeneic APC (30). Other investigators recently reported that $CD26^{high}$ T cells contain T_H17 cells, and that $CD26^{high}$ T_H17 cells are enriched in inflamed tissues including rheumatoid arthritis (RA) and inflammatory bowel diseases (IBD) (31). These accumulating data strongly suggest that CD26-mediated costimulation plays an important role in memory response to recall antigens, and that blockade of CD26 costimulation may be an effective therapeutic strategy for immune disorders including GVHD or autoimmune diseases.

3.1.2. Newly established humanized murine model of cGVHD

We previously analyzed a humanized murine aGVHD model involving mice transplanted with human adult peripheral blood lymphocytes (PBL), and showed that liver and skin were predominantly involved as target organs in this model of aGVHD, which was clearly impeded by the administration of humanized anti-CD26 monoclonal antibody (mAb) (32). Our data suggest that $CD26^+$ T cells play an effector role in this aGVHD model. However, since the mice studied in our previous work succumbed to aGVHD around 4 weeks after transplantation of human adult PBL, this early-onset model of aGVHD does not permit the assessment of longer term consequences of interventional therapies, such as their effect on the development of OB, a form of cGVHD of the lung.

In contrast to adult PBL, human umbilical cord blood (HuCB) lymphocytes have been reported to be immature, predominantly consisting of $CD45RA^+$ naïve cells (33, 34). We previously showed that, while all HuCB CD4 T cells constitutively express CD26, CD26-mediated costimulation was considerably attenuated in HuCB CD4 T cells, compared to the robust activation via CD26 costimulation of adult PBL (34). These findings provided further insights into the cellular mechanisms of immature immune response in HuCB. Based on these findings, we hypothesized that HuCB naïve CD4 T cells gradually acquire a xenogeneic response via attenuated stimulatory signaling with indolent inflammation in the target organs, leading eventually to chronic inflammatory changes. We therefore sought to develop a humanized murine pulmonary cGVHD model utilizing HuCB donor cells, and to overcome the limitations seen in the humanized murine aGVHD model such as vigorous activation of all engrafted T cells and extensive loss of B cell maturation and activation (35, 36).

We first attempted to establish a humanized murine model utilizing NOD/Shi-*scid*IL2rγ^{null} (NOG) mice as recipients and HuCB as donor cells (37). Whole CB transplant mice exhibited clinical signs/symptoms of GVHD as early as 4 weeks post-transplant, and demonstrated significantly decreased survival rate. The lung of whole CB transplant mice showed perivascular and subepithelial inflammation and fibrotic narrowing of the bronchiole. Skin of whole CB transplant mice manifested fat loss, follicular drop-out and sclerosis of the reticular dermis in the presence of apoptosis of the basilar keratinocytes while the liver exhibited portal fibrosis and cholestasis. These findings indicate that whole CB transplant mice develop pulmonary cGVDH as well as concomitant active GVHD in skin and liver. Taken together, our data demonstrate that the lung of whole CB transplant mice exhibits OB as manifestation of pulmonary cGVHD.

3.1.3. IL-26 contributes to the pathophysiology of pulmonary cGVHD

To determine the potential cellular mechanisms involved in the pathogenesis of pulmonary cGVHD, we next analyzed the composition of donor-derived human lymphocytes in the GVHD lung. Utilizing flow cytometric analysis for cell suspension isolated from the lung specimens, donor-derived human $CD3^+$ cells were found to be the predominant cell type observed in the

lung of whole CB transplant mice, comprising more than 99% of the lymphocyte population. Moreover, the human CD4 T cell subset was observed to be the predominant cell type compared to CD8 T cells in the lung of whole CB transplant mice. We next analyzed the expression profile of mRNAs of various inflammatory cytokines in human CD4 T cells isolated from the lung of whole CB transplant mice. We found that *IFNG*, *IL17A, IL21* and *IL26* were significantly increased over the course of GVHD development following whole CB transplantation, while *IL2*, *TNF* (TNF-α), *IL4*, *IL6* and *IL10* were decreased. In addition, substantial increases were seen in levels of *IFNG* and *IL26*, with *IL17A* and *IL21* remained at a low level. It has been reported that IFN-γ is produced by T_H1 cells (6), while IL-17A and IL-26 are produced by T_H17 cells (38, 39). Since both T_H1 and T_H17 cells strongly express CD26 (6, 31), we next analyzed the expression level of CD26/DPP4, finding that *DPP4* mRNA expression in human CD4 T cells infiltrating in the lung of mice with OB was significantly increased. These findings regarding mRNA expression levels were further supported by enzyme-linked immunosorbent assay (ELISA) studies examining protein levels in sera of recipient mice. To determine whether these cytokines were produced by the infiltrating human $CD26^+$CD4 T cells, we next conducted flow cytometric analyses of lymphocytes isolated from the lung of the recipient mice. Levels of human IFN-γ^+ or IL-26^+CD26^+CD4 T cells were significantly increased in whole CB transplant mice. Multicolor-staining flow cytometric studies showed that $CD26^+$CD4 T cells in the lung of whole CB transplant mice predominantly produced IL-26 rather than IFN-γ. In addition, while $CD26^+$IFN-γ^+CD4 cells exclusively expressed IL-26, $CD26^+$IL-26^+CD4 cells were predominantly IFN-γ-negative cells, and IL-$17A^+$ cells were exclusively IL-26-negative. These data suggest that $CD26^+$CD4 T cells in the lung of mice with OB express IL-26 as well as IFN-γ but do not belong to the T_H17 cell population.

To further extend the above *in vitro* results to an *in vivo* system, we analyzed the lung of murine alloreactive GVHD using human *IL26* transgenic (Tg) mice. For this purpose, we used mice carrying human *IFNG* and *IL26* transgene (190-*IFNG* Tg mice) or mice carrying human *IFNG* transgene with deleting *IL26* transcription (ΔCNS-77 Tg mice). 190-*IFNG* Tg mice exhibited production of IL-26 by CD4 T cells under T_H1- or T_H17-polarizing conditions, while expression of IL-26 was completely abrogated in ΔCNS-77 Tg mice (38). In addition, production of IFN-γ by T or NK cells was equivalent in both 190-*IFNG* Tg and ΔCNS-77 Tg mice (40). Histologic examinations of the lung of recipient NOG mice deriving from parental C57BL/6 (B6 WT) mice or ΔCNS-77 Tg mice showed peribronchial infiltration and cuffing denoting GVHD, while collagen deposits were not detected by Mallory staining, and IL-26^+ cells were not detected. On the other hand, the lung of recipient NOG mice deriving from 190-*IFNG* Tg mice showed peribronchial infiltration and cuffing denoting GVHD with collagen deposition and IL-26^+ cell infiltration. These results suggest that human IL-26, but not human IFN-γ, plays a critical role in pulmonary fibrosis associated with lung cGVHD.

3.1.4. IL-26 production via CD26-mediated T cell costimulation

To test whether human CD4 T cells produce IL-26 following CD26 costimulation, we conducted *in vitro* costimulation experiments using HuCB CD4 T cells and analyzed expression of various inflammatory cytokines. We found that levels of *IL26* and *DPP4* were significantly increased following CD26 costimulation compared with CD28 costimulation. We next conducted costimulation experiments evaluating dose and time kinetics using the CD26 costimulatory ligand Cav-Ig as well as anti-CD26 or anti-CD28 mAbs. We showed that production of IL-26 was increased following CD26 costimulation with Cav-Ig or anti-CD26 mAb in dose- and time-dependent manners, while a slight increase in IL-26 level was observed following CD28 costimulation only at higher doses of mAb and longer stimulation periods. Blocking experiments were then performed for further confirmation, showing that IL-26 production induced by Cav-Ig or anti-CD26 mAb was clearly inhibited by treatment with soluble Cav-Ig in a dose-dependent manner, while no change was observed with CD28 costimulation. These findings strongly suggest that production of IL-26 by HuCB CD4 T cells is regulated via CD26-mediated costimulation. Moreover, since the functional sequences of the N-terminal of caveolin-1 are highly conserved between human and mouse (41) allowing for the capability to bind human CD26 as a costimulatory ligand, it is conceivable that donor HuCB T cells transferred into mice were activated via CD26 costimulation triggered by murine caveolin-1. In fact, using polyclonal antibody recognizing the N-terminal of both human and murine caveolin-1, expression of caveolin-1 was detected in endothelial cells and macrophage-like cells of OB-like lesions in cGVHD lung. Taken together, CD26-mediated IL-26 production triggered by caveolin-1 is identified as a possible therapeutic target in cGVHD using HuCB NOG mice.

3.1.5. Prevention of lung cGVHD development by Cav-Ig administration

Given the role of CD26 costimulation in IL-26 production and IL-26 regulation of collagen production, we therefore sought to determine whether disruption of CD26 costimulation by a blocking reagent, Cav-Ig, prolonged survival of the recipient mice associated with a reduction in the incidence of OB. Recipients treated with Cav-Ig survived for 7 months without any clinical findings of cGVHD. Meanwhile, the survival rate of recipient mice treated with control Ig was significantly reduced, with clinical signs/symptoms of cGVHD. Human cells were engrafted similarly in both groups. Histologic examinations of the lung showed the development of OB in the control Ig cohort, while the lung of Cav-Ig recipient mice displayed normal appearances with none having positive pathology scores. These effects of Cav-Ig were also observed in other GVHD-target organs such as the skin and liver. Moreover, collagen contents in the lung were reduced in Cav-Ig administered-recipients. Taken together, the above results support the notion that Cav-Ig administration prevents the development of pulmonary cGVHD in whole CB transplant mice by decreasing the number of IL-26^+CD26^+CD4 T cells.

3.1.6. Treatment with Cav-Ig preserves GVL capability

Since GVHD and GVL effect are highly linked immune reactions (42), we evaluated the potential influence of Cav-Ig treatment on GVL effect. For this purpose, cohorts of Cav-Ig or control Ig treated recipient mice of whole CB transplant were irradiated at sublethal doses and then injected intravenously with luciferase-transfected A20 (A20-luc) cells 1 day prior to whole CB transplantation to allow for dissemination of tumor cells. The next day following transplantation, treatment with Cav-Ig or control Ig thrice a week began on day +1 until day +28. Mice inoculated with A20 cells alone all died of tumor progression within 6 weeks. Recipients treated with control Ig exhibited clinical evidence of GVHD such as weight loss and ruffled fur and died of GVHD without tumor progression in 13 weeks. In contrast, recipient mice treated with Cav-Ig displayed significantly prolonged survival without involvement of A20-luc cells. To better characterize the potency of the GVL effect, we repeated these studies with injection of A20-luc cells on day +28 after whole CB transplantation to allow for acquisition of immunosuppression by Cav-Ig treatment. Mice inoculated with A20 cells alone all died of tumor progression within 2 weeks after tumor inoculation. Recipient mice treated with control Ig demonstrated clinical evidence of GVHD such as weight loss and ruffled fur and died of GVHD without tumor progression within 13 weeks after transplantation. In contrast, recipients treated with Cav-Ig exhibited significantly prolonged survival without involvement of A20-luc cells. Collectively, these results demonstrate that Cav-Ig treatment of recipient mice of whole CB transplant was effective in reducing the symptoms of cGVHD without a concomitant loss of the GVL effect.

3.1.7. Role of CD26 in cGVHD

While the human CD26 amino acid (AA) sequence has 85% AA identity with the mouse CD26 (43), the mouse CD26 has different biologic properties from the human CD26, including the fact that the mouse CD26 is not a T cell activation marker, and does not bind to adenosine deaminase (ADA) (43, 44). Therefore, humanized murine models need to be developed to explore the role of CD26-mediated costimulation in cGVHD. With relevance as a costimulatory ligand for human CD26, human caveolin-1 has 95% AA identity with the mouse caveolin-1 (41), and the binding regions of the mouse caveolin-1 for human CD26 are well conserved. Therefore, costimulatory activation of human T cells in NOG mice can occur via CD26-caveolin-1 interaction. Moreover, the N-terminal domain is present in the outer cell surface during the antigen presenting process (27), and caveolin-1 forms homo-dimer or homo-oligomer via its N-terminal domain (41). These collective data suggest that the administered Cav-Ig binds to the N-terminal of caveolin-1 on the cell surface of APCs as well as to CD26 in T cells, leading to suppression of cGVHD in HuCB-NOG mice via blockade of CD26-caveolin-1 interaction. Conclusively, our work demonstrates that caveolin-1 blockade controls cGVHD by suppressing the immune functions of donor-derived T cells and decreasing IL-26 production. Moreover, IL-26^{+}CD26^{+}CD4 T cell infiltration appears to play a significant role in cGVHD of the lung and skin. While complete suppression of cGVHD with current interventional strategies represents a difficult challenge at the present time, our data demonstrate that control of cGVHD clinical findings can be achieved in a murine experimental system by regulating IL-26^{+}CD26^{+}CD4 T cells with Cav-Ig. Our work also suggests that Cav-Ig treatment may be a novel therapeutic approach for chronic inflammatory diseases, including RA and IBD, in which IL-26 plays an important role.

3.2. Middle East respiratory syndrome coronavirus

3.2.1. Current efforts against Middle East respiratory syndrome coronavirus

Middle East respiratory syndrome coronavirus (MERS-CoV) was first identified in a 60-year-old patient in June 2012 who presented with acute pneumonia, followed by acute respiratory distress syndrome and renal failure with a fatal outcome (45). Between 2012 and August 28, 2017, 2067 laboratory-confirmed cases of MERS-CoV infection were reported to the World Health Organization (WHO), which has notified of at least 720 deaths (around 35% fatality rate) related to MERS-CoV since September 2012 (46). Efforts to develop effective preventive and therapeutic intervention strategies are currently ongoing. Several treatment modalities have been investigated to develop effective therapies against MERS-CoV, including interferon, ribavirin, cyclosporin A, protease inhibitors, convalescent plasma and immunoglobulins. Several promising anti-MERS-CoV therapeutic agents have recently been reviewed extensively (47), while broad spectrum antiviral agents might not be sufficient to treat severe MERS-CoV patients because of its limited effective therapeutic window of opportunity (48).

An alternative approach using prophylactic regimens would be theoretically suitable to limit the spread of MERS-CoV. This scenario includes MERS-CoV vaccine and neutralizing MERS-CoV-specific mAb (48). The MERS-CoV genome encodes for 16 non-structural proteins (nsp1-16) and 4 structural proteins, the spike (S), envelope (E), membrane (M), and nucleocapsid (N) (49). The viral structural proteins, S and N, show the highest immunogenicity (50). While both S and N proteins induce T-cell responses, neutralizing antibodies are almost solely directed against the S protein, with the receptor binding domain (RBD) being the major immunodominant region (51). These great challenges have been extensively reviewed in previously published papers (48, 52).

Recent reports indicated that the spike protein S1 of MERS-CoV is required for viral entry into human host cells (53-55), using CD26/DPPIV as a functional receptor (56). Inhibiting virus entry into host cells could also be achieved by targeting the host receptor CD26/DPPIV. While inhibitors of the CD26/DPPIV enzymatic activity are used clinically to treat type 2 diabetes patients, commercially available DPPIV inhibitors would not serve this purpose since these agents have been shown not to inhibit binding of the RBD of MERS-CoV to CD26/DPPIV (57). We previously showed that human CD26 is a binding protein for ADA (58). Currently, it is known that there are two isoforms of ADA, ADA1 and ADA2 (59). ADA1 is particularly present in lymphocytes and macrophages, while ADA2 is found predominantly in the serum and other body fluids including pleural effusion (59). CD26/DPPIV binds to ADA1, but not ADA2 (58, 60, 61). The crystal structure of CD26/DPPIV and the S protein

of MERS-CoV allowed for visualization of the interacting AA in both proteins. However, our *in vitro* experiments showed that blockade of MERS-CoV binding to CD26/DPPIV by ADA1 is incomplete (62). Therefore, mAbs blocking CD26/DPPIV binding to the RBD of MERS-CoV needs to be developed.

3.2.2. CD26/DPPIV is a functional receptor for MERS-CoV entry into host cells

CD26/DPPIV, a host receptor for MERS-CoV, is conserved among different species such as bats and humans, partially explaining the large host range of MERS-CoV (63). In addition to being widely expressed in most cell types including T lymphocytes, bronchial mucosa or the brush border of proximal tubules, CD26/DPPIV exists in systemic circulation as soluble form (13). This distribution of CD26 may play a role in the systemic dissemination of MERS-CoV infection in human (64-66). Therefore, an effective therapy for MERS-CoV is needed not only to block the entry of MERS-CoV into CD26-expressing organs such as the respiratory system, kidney, liver or intestine, but also to eliminate circulating MERS-CoV. In this regard, manipulation of CD26/DPPIV levels or the development of inhibitors that target the interaction between the MERS-CoV S1 domain and its receptor may provide therapeutic opportunities to combat MERS-CoV infection. More recently, the RBD in the S protein was mapped to a 231-AA fragment of MERS-CoV S proteins (residues 358-588) (51).

3.2.3. Identification of specific anti-CD26 mAb clone for blocking MERS-CoV

We have recently mapped MERS-CoV S protein-binding regions in human CD26 molecule and demonstrated that anti-CD26 mAbs, which had been developed in our laboratory, effectively blocked the interaction between the spike protein and CD26, thereby neutralizing MERS-CoV infectivity. To determine the specific CD26 domain involved in MERS-CoV infection, we chose six different clones of anti-CD26 mAbs (4G8, 1F7, 2F9, 16D4B, 9C11, 14D10), and the humanized anti-CD26 mAb YS110, which recognize 6 distinct epitopes of the CD26 molecule (67, 68), to conduct MERS-CoV S1-Fc (S1 domain of MERS-CoV fused to the Fc region of human IgG) binding inhibition assays (62). 2F9 inhibited fully binding of MERS-CoV S1-Fc to JKT-hCD26WT (Jurkat cells transfected with full-length human CD26/DPPIV), while other anti-CD26 mAbs demonstrated certain levels of inhibition (1F7, or YS110) or no significant inhibition (4G8, 16D4B, 9C11 or 14D10). These results strongly suggest that the anti-CD26 mAb 2F9 has better therapeutic potential than recombinant MERS-CoV S1-Fc in preventing viral entry into susceptible cells, and that 1F7 or YS110 also block MERS-CoV infection.

Moreover, we have characterized the CD26 epitope involved in MERS-CoV S1-Fc binding to CD26 through the use of various CD26 mutants with deletion in the C-terminal extracellular region, since CD26 is a type II transmembrane protein (9, 29). Our biological experiments on binding regions of CD26 to MERS-CoV using mAbs showed results comparable to those obtained from crystal structure analysis (55, 69), which are summarized in a schematic diagram of human CD26 at 1-449th AAs (Figure 1). Our observations strongly suggest that the main binding regions of CD26 to MERS-CoV appear to be close to the 358th AAs recognized by 2F9, and the regions of CD26 defined by 1F7 and YS110 (248-358th AAs) are also partially involved in MERS-CoV binding.

To determine whether treatment with anti-CD26 mAbs 2F9 as well as 1F7 and YS110 could inhibit MERS-CoV infection, susceptible cells, Huh-7, were pre-incubated with various anti-CD26 mAbs prior to inoculation with the virus (62). In this experimental system, infection was almost completely blocked by 2F9 but not by control IgG or several other anti-CD26 mAbs recognizing other epitopes (4G8, 16D4B, 14D10 or 9C11). Moreover, the anti-CD26 mAbs 1F7 and YS110 considerably inhibited MERS-CoV infection of Huh-7 cells. These results demonstrate that 2F9 inhibits MERS-CoV entry, and therefore can potentially be developed as a preventive or therapeutic agent for MERS-CoV infection in the clinical setting. More importantly, the humanized anti-CD26 mAb YS110 has been evaluated in patients with CD26-expressing cancers in our recent first-in-human (FIH) phase I clinical trial (70). Since no apparent adverse effects of YS110 have been reported besides transient and tolerable injection reactions, YS110 may be an immediate therapeutic candidate for clinical use as potential treatment for MERS-CoV infection.

3.3. Psoriatic pruritus

3.3.1. CD26/DPPIV and psoriasis

Psoriasis (PSO) is one of the most common inflammatory skin diseases, found in about 1-3% of the world general population (71). For a long time, PSO had been considered as a non-pruritic dermatitis. However, within the past 30 years, a number of studies have demonstrated that approximately 60-90% of patients with PSO suffer from pruritus (71-76). Pruritus is an important symptom of PSO. Despite the fact that several studies have been undertaken to investigate the pathogenesis of pruritus in PSO, many aspects have not yet been studied (71, 77). Therefore, the pathogenesis of this symptom is far from being well-understood and, as a consequence, the therapy of pruritic psoriatic patients still remains a significant challenge for clinicians (78). It has been demonstrated that DPPIV is expressed at high levels on keratinocytes and that DPPIV inhibition suppresses keratinocyte proliferation *in vitro*, and restores partially keratinocyte differentiation *in vivo* (79). Moreover, it has been reported that DPPIV is expressed on keratinocytes and its activity is upregulated in PSO (80, 81), findings which support a potential role for DPPIV enzyme activity in the pathogenesis of PSO. While other investigators have reported a significant improvement in disease severity in PSO patients treated with a DPPIV inhibitor (82, 83), the precise mechanisms involved in DPPIV-mediated regulation of PSO have not been elucidated (84). Recent report showed that T-cell bound expression of CD26/DPPIV in psoriatic skin was explicitly present, albeit in small quantities (81). One hypothesis of potential effect of DPPIV in PSO is that T cell activation mediated by DPPIV is associated with the pathogenesis of PSO (85). Cytokines and chemokines represent the third

key player in the psoriatic chronic immune response (86). They are considered as mediators responsible for activation and recruitment of infiltrating leukocytes and therefore play a crucial role in the development and persistence of psoriatic skin lesions (87). DPPIV likely plays a pivotal role in the processing of these molecules (84). The extracellular protease domain of DPPIV (both on keratinocytes and T cells) can cleave dipeptides from the amino terminus of proteins, such as cytokines and chemokines, which are abundantly present in a chronic immune response in PSO, resulting in alterations in receptor specificity and subsequent modifications of biological activity. Taken together, it is conceivable that PSO is a disease involving the complex interplay among activated T cells, keratinocytes and cytokines, and that DPPIV has a key regulatory role in the interactions of these three disease components.

3.3.2. Elevation of sCD26 and DPPIV enzyme in sera of PSO patients

To determine whether serum soluble CD26 (sCD26) and soluble DPPIV (sDPPIV) enzyme play a role in PSO, we evaluated levels of sCD26 and sDPPIV enzyme activity in sera of patients with PSO (88). For this purpose, we performed our in-house capture assay method using anti-human CD26 mAb as a capture antibody for detecting DPPIV enzyme activity specific to sCD26 (89). Since commercially available DPPIV enzyme assay kits measure DPPIV activity in whole serum, but not in captured sCD26 molecules from the samples, it is possible that DPPIV-like peptidase activity other than that possessed by the captured sCD26 molecules was measured, leading to an overestimate of the DPPIV activity in the samples (90). Analyses of serum samples obtained from 18 healthy adult volunteers and 48 PSO patients demonstrated that serum sCD26 concentration of PSO patients was significantly higher than that of healthy adults. Moreover, serum levels of sDPPIV enzyme activity were also significantly higher in patients with PSO compared with healthy adult controls. These data suggest that DPPIV enzyme activity is increased in sera of patients with PSO, which is linked to a concomitant increase in sCD26 in the same patient population. These observations also suggest that DPPIV enzyme plays a role in the pathogenesis of PSO.

3.3.3. Increased pruritus by truncation of substance P, a ligand for CD26/DPPIV

Among various mediators of pruritus investigated in inflammatory skin diseases, substance P (SP) is a key molecule in an itch sensory nerve (91-93), consisting of 11 AA residues with dual DPPIV cleavage sites at its N-terminal position. In fact, DPPIV enzyme digests full-length SP(1-11) resulting in a truncated form of SP(5-11), an activity inhibited by the presence of the DPPIV enzyme inhibitor sitagliptin (88). Moreover, we observed that levels of SP degraded by DPPIV were increased in sera of patients with PSO. Taken together with the above data regarding an increase of sCD26/DPPIV levels in PSO patients, these results also suggest that the increase in DPPIV activity appears to play an important role in PSO by truncating SP.

We next utilized an itchy mouse model by intradermal injection (i.d.) of recombinant SP and quantified scratching behavior in mice to determine an itchy symptom. Mice treated with SP(5-11) i.d. demonstrated a significant increase in scratching behavior, compared with mice receiving control solvent or mice receiving full-length SP(1-11). On the other hand, scratching behavior in SP(1-11) i.d. mice was significantly decreased in mice treated with the DPPIV inhibitor sitagliptin. Furthermore, SP-induced scratching behavior was significantly attenuated in CD26/DPPIV knockout (CD26KO) mice compared with that observed in B6 WT mice. Our data suggest that truncated form of SP cleaved by DPPIV enzyme increases an itch sensation and that SP-induced itch sensation is attenuated by inhibition of the DPPIV activity.

To further determine that DPPIV inhibition affects pruritus, we evaluated scratching behavior utilizing an imiquimod (IMQ)-induced psoriatic itch model (94, 95). Serum levels of truncated form of SP were significantly increased in IMQ-treated mice compared with control cream-treated mice. Moreover, scratching behavior was significantly increased in IMQ-treated mice than control cream-treated mice. These data indicate that IMQ induces psoriatic itchy skin lesions in mice associated with an increase in the truncation of SP. We next analyzed the frequencies of itch scratching behavior following DPPIV inhibitor administration. IMQ-treated mice receiving sitagliptin showed significant decrease of scratching behavior compared with IMQ-treated mice receiving control solvent. Meanwhile, there was no change in scratching behavior between control cream-treated mice receiving sitagliptin or control saline, with baseline levels of scratching behavior in both cohorts. Taken together, our data suggest that treatment with the DPPIV inhibitor sitagliptin attenuates psoriatic itch sensation via a decrease in the truncated form of SP.

Previous studies have reported that serum levels of SP were decreased in patients with PSO (96-98). Meanwhile, since SP is cleaved by DPPIV enzyme and DPPIV enzyme activity is increased in PSO (88), it is important for a detailed understanding of the role of SP in PSO to precisely measure the truncated form of SP separately from full-length SP. In our recent study, we evaluated full-length SP(1-11) and truncated forms of SP and demonstrated that there was no change in the serum levels of full-length SP(1-11), SP(2-11) and SP(3-11) between PSO and healthy adult controls (88). However, we found that DPPIV enzyme activity and the truncated form of SP were significantly increased in PSO, and that the truncated form of SP(5-11) resulting from DPPIV enzyme activity is associated with an increase in itch sensation. In the IMQ-induced PSO model, the truncated form of SP was significantly increased in sera compared with control mice, and scratching behavior was decreased by administration of sitagliptin. On the other hand, there were no differences in serum levels of DPPIV enzyme activity between IMQ and control cream-treated mice. It is conceivable that the persistent existence of psoriatic skin lesions may be required for the increased serum levels of DPPIV enzyme activity seen in PSO patients, and that SP truncation may result from the increased levels of DPPIV enzyme activity in skin lesions rather than in the circulation (80, 99, 100). Our recent study has conclusively demonstrated that increase in DPPIV enzyme activity exacerbates pruritus in PSO, and that inhibition of DPPIV enzyme reduces

severity of itch scratching behavior. Moreover, our results suggest that DPPIV inhibitors are useful as therapeutic agents for pruritus including PSO.

4. CANCERS

4.1. Novel mechanism of CD26/DPPIV in cancer immunology

4.1.1. Anti-tumor effect of CXCL10-mediated CXCR3$^+$ lymphocyte via DPPIV inhibition

CD26/DPPIV regulates the activities of a number of cytokines and chemokines. However, direct *in vivo* evidence for a role for CD26 in tumor biology and its interaction with the tumor microenvironment (TME) has not yet been reported. Recent work has demonstrated clearly the interaction between DPPIV and substrate CXCL10, as well as the functional role of DPPIV-mediated post-translational modification of chemokines in regulating tumor immunity (101). Preservation of the full length, bioactive CXCL10 by DPPIV inhibition results in increased level of CXCR3$^+$ effector T cells in the TME and subsequent tumor growth reduction. CXCR3 has been shown to be a functional receptor for CXCL10 (102). Importantly, the combination of DPPIV inhibition and checkpoint blockade therapy remarkably augments the efficacy of naturally occurring and immunotherapy-based tumor immunity. These investigators therefore provide the direct evidence of DPPIV as an *in vivo* regulator of CXCL10-mediated T cell trafficking with relevance for tumor immunity and immunotherapy (Figure 2). The TME consists of numerous cell types along with the neoplastic cells. Among them are the effector lymphocytes capable of infiltrating into the tumor sites that are specifically required for anti-cancer immune response (103). CXCL10 is a chemoattractant for immune cells such as monocytes, T cells and NK cells and is secreted from a variety of cells in response to IFN-γ, including monocytes, neutrophils, eosinophils, epithelial cells, endothelial cells, fibroblasts and keratinocytes (104). CXCL10 appears to have a dual role on tumor growth, with its proliferative or anti-proliferative activity being cell-type-dependent as a result of differences in the subtype of its receptor CXCR3 (104). CXCR3 is rapidly induced on naïve T cells following activation and preferentially remains highly expressed on T_H1-type CD4$^+$ cells and CD8$^+$ cytotoxic T lymphocytes (CTL), resulting in enhancement of T cell migration to facilitate tumor immune responses (105). Although strong T_H1 and CTL responses in the TME are beneficial for tumor suppression, these responses are counterbalanced to prevent unwanted tissue damage and immunopathology by disrupting the proinflammatory loop. CXCR3$^+$ T_{reg} has been recently identified (106) , as IFN-γ signaling activates the T_H1 transcription factor *T-bet*, which in turn promotes CXCR3 expression to induce T_H1-specific T_{reg} in the inflammatory sites. Moreover, CXCR3 is a marker of CD8$^+$ IL-10-producing cells with suppressive activity in both mice and human (107). The exact factors determining whether CXCR3$^+$ effector T cells and CXCR3$^+$ regulatory lymphocytes will oppose or cooperate with each other during the tumor growth process *in vivo* remain to be elucidated.

4.1.2. Immune checkpoint mechanism via CD26/DPPIV

Although the cellular and molecular mechanisms involved in CD26-mediated T cell activation have been extensively evaluated by our group and others (4-6, 9, 13, 90), potential negative feedback mechanisms to regulate CD26-mediated activation still remain to be elucidated. Utilizing human PBL, we found that production of IL-10 by CD4$^+$ T cells is preferentially increased following CD26-mediated costimulation compared with CD28-mediated costimulation (108). IL-21 production was also greatly enhanced in the late phase of CD26 costimulation. On the other hand, production of IL-2, IL-5 or TNF-α was much lower following CD26 costimulation than CD28 costimulation. In contrast, no difference in the production of IL-17A, IFN-γ, or IL-4 was observed following CD26 or CD28-mediated costimulation. These data indicate that CD26 and CD28 costimulation of CD4$^+$ T cells results in different cytokine production profiles, with IL-10 production being preferentially enhanced following CD26 costimulation. Furthermore, we found that both the cell surface and intracellular expression of LAG3 (lymphocyte activation gene-3) was clearly enhanced with increasing doses of anti-CD26 mAb, and that CD26-induced enhancement of LAG3 was more pronounced than the effect of CD28-mediated costimulation. On the other hand, both CD26 and CD28-mediated costimulation enhanced the expression of CTLA-4 (cytotoxic T-lymphocyte-associated antigen 4) and FOXP3 (forkhead box protein P3), with no significant difference being detected between these two costimulatory pathways. In contrast with CD28 costimulation, LAP (latency associated protein) complexed with TGF-β1 was hardly induced following CD26 costimulation. We showed that all the CD4$^+$ T cells expressed LAG3 following CD26 or CD28 costimulation, and that no difference was observed in the percentage of LAG3 expressing cells, while the expression intensity of LAG3 after CD26-mediated costimulation was significantly higher than after CD28-mediated costimulation. LAG3 serves as a marker of IL-10 producing T_{reg} (109), and binds to major histocompatibility complex (MHC) class II molecules with higher affinity than CD4, leading to transduction of inhibitory signals for both T cells and APCs (110, 111). Therefore, our data strongly suggest that signaling events via CD26 may induce the development of CD4$^+$ T cells to a Type 1 regulatory T cells (Tr1)-like phenotype. By expression analysis with Western blotting and quantitative real-time polymerase chain reaction (RT-PCR) experiments and by cell functional analysis utilizing chemical inhibitors and small interfering RNA (siRNA) experiments, we showed that co-engagement of CD3 and CD26 induces preferential production of IL-10 in human CD4$^+$ T cells, mediated through NFAT (nuclear factor of activated T cells) and Raf (rapidly accelerated fibrosarcoma)-MEK (mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase)-ERK pathways (108). High level of early growth response 2 (EGR2) is also induced following CD26 costimulation, possibly via NFAT and AP-1 (activator protein-1)-mediated signaling, and knock down of EGR2 leads to decreased IL-10 production. Taken together, these observations strongly suggest that CD26-mediated costimulation of CD4$^+$ T cells results in enhanced NFAT/AP-1-dependent EGR2 expression, which is associated with the preferential production of IL-10. Finally, we demonstrated that CD3/CD26-stimulated CD4$^+$ T cells clearly suppress

proliferative activity and effector cytokine production of bystander T cells in an IL-10-dependent manner (108). Collectively, our results above suggest that CD3/CD26 costimulation induces the development of human Tr1-like cells from $CD4^+$ T cells with high level of IL-10 production and LAG3 expression. Preclinical models showed that antibody-mediated blocking of LAG3 as potential anti-cancer therapy led to enhanced activation of antigen-specific T cells at the tumor sites and disruption of tumor growth (112). Moreover, anti-LAG3/anti-PD-1 (programmed cell death 1) antibody treatment cured most mice of established tumors that were largely resistant to single antibody treatment (112). Taken together, it is conceivable that CD26 itself may function as an inhibitory molecule of an immune checkpoint system in certain disease conditions, similar to LAG3 or PD-1.

4.2. Malignant pleural mesothelioma

4.2.1. FIH phase I clinical trial of humanized anti-CD26 mAb

Our previous work analyzing extracellular matrix (ECM) interactions and intracellular signaling events demonstrated that the malignant mesothelial cell line JMN expresses CD26 (113). Our recent in-depth studies of CD26 expression in malignant pleural mesothelioma (MPM) revealed that CD26 is preferentially expressed in MPM cells but not in normal mesothelial cells (114, 115). These intriguing findings propelled our development of CD26-targeted therapy for MPM. For this purpose, we had developed a novel humanized anti-CD26 mAb, namely YS110. YS110 is a recombinant DNA-derived humanized mAb that selectively binds with high affinity to the extracellular domain of CD26. The antibody is an $IgG_1\kappa$ with a molecular weight of 144 kDa and was humanized via an *in silico* design based on the AA sequence of anti-human CD26 murine mAb (14D10), which inhibited tumor cell growth, migration and invasion, and enhanced survival of mouse xenograft models (116). YS110 is produced by fermentation in CHO (Chinese hamster ovary) mammalian cell suspension culture with the Glutamine Synthetase Expression System. *In vitro* pharmacologic evaluation of YS110 demonstrated its selective binding to human CD26 on a number of human cancer cell lines and tissues, with no apparent effect on immune activation and no inhibition of DPPIV activity, while exhibiting direct cytotoxic effect on certain human CD26-positive cancer cell lines. Moreover, our *in vitro* data indicated that YS110 induces cell lysis of MPM cells via antibody-dependent cellular cytotoxicity (ADCC) in addition to its direct anti-tumor effect via cyclin-dependent kinase inhibitor (CDKI) $p27^{kip1}$ and $p21^{cip1}$ accumulation (68, 117). *In vivo* experiments with mouse xenograft models involving human MPM cells demonstrated that YS110 treatment drastically inhibits tumor growth in tumor-bearing mice and reduces formation of metastases, resulting in enhanced survival (68). Our data strongly suggest that YS110 may have potential clinical use as a novel cancer therapeutic agent for CD26-positive malignant mesothelioma.

In addition to our robust *in vitro* and *in vivo* data on antibody-mediated dose-dependent tumor growth inhibition, YS110 exhibited excellent safety and pharmacological profiles in non-human primate models using single and repeated increasing intravenous doses. Considering the lack of T cell proliferation and cytokine production *in vitro*, YS110 was therefore considered not to have an agonistic nor activating effect on human CD26-positive lymphocytes. Other key safety findings were obtained from studies involving cynomolgus monkeys which express YS110-reactive CD26 molecules, with similar tissue distribution profiles and expression levels to human CD26. We therefore conducted the FIH clinical trial of YS110 for patients with MPM and other CD26-positive solid tumors (70). Thirty-three heavily pre-treated patients with CD26-positive cancers including 22 malignant mesothelioma, 10 renal cell carcinoma (RCC) and 1 urothelial carcinoma underwent YS110 administration. A total of 232 infusions (median 3 (range 1-30)) of YS110 were administered across 6 dose levels ranging from 0.1. to 6.0. mg/kg. Maximum tolerated dose (MTD) was not reached and 2 dose limiting toxicities (DLTs) (1 patient with grade 3 anaphylactic reaction at 1.0. mg/kg and 1 patient with grade 3 allergic reaction at 2.0. mg/kg) were reported with complete resolution following dose omission. Subsequent use of systemic steroid prophylaxis and exclusion of patients with significant allergy histories improved safety profile. No dose-dependent adverse events were observed. Blood exposure pharmacokinetics parameters (AUC and C_{max}) increased in proportion with the dose. Cytokines and immunophenotyping assays indicated CD26 target modulation with no occurrence of infectious nor autoimmune disease complications. These results demonstrated that YS110 is tolerable in human subjects. A secondary objective of this FIH study was to evaluate the potential antitumor activity of YS110 according to RECIST 1.0. (response evaluation criteria in solid tumors) criteria (or modified RECIST criteria for mesothelioma). No objective response was achieved in any of the treated patients. However, stable disease as the best overall response was observed in 13 out of the 26 valuable patients on Day 43 of the first cycle (1 at 0.1. mg/kg, 2 at 0.4. mg/kg, 7 at 2.0. mg/kg, 1 at 4.0. mg/kg and 2 at 6.0. mg/kg). Prolonged stabilization with 26 weeks or more was observed in 7 out of 13 stable disease patients who have received a total of 143 (5 to 30 infusions/patients) infusions with a median PFS (progression-free survival) of 33 weeks (26 to 57 weeks). This FIH study conclusively demonstrated that YS110 exhibits a favorable safety profile and substantial clinical activity in heavily pretreated CD26-positive MPM patients who had previously progressed on conventional standard chemotherapies. Further clinical trial of YS110 for MPM is in progress worldwide (118).

4.2.2. DPPIV enzyme activity and efficacy of YS110

The FIH clinical study of YS110 revealed that an increase in YS110 infusion dose was associated with decreased serum sCD26 level, particularly in cohorts 4-6 (2.0. to 6.0. mg/kg), with an approximately 80% decrease in sCD26 level (70). Moreover, since sCD26 level reflects DPPIV enzyme activity in sera (119), similar reduction in DPPIV enzyme activity was observed, again particularly in patients in cohorts 4-6. Although DPPIV inhibitors are clinically used as oral hypoglycemic agents (120), hypoglycemia was not observed during YS110 administration. Of note is the fact that greater than 80% inhibition of serum DPPIV activity was obtained 24 hours after oral administration of clinically available DPPIV inhibitors (drug information published by manufacturers of sitagliptin, vildagliptin, saxagliptin and *etc.*), a level of inhibition comparable to that seen in patients treated with YS110. Our current data would therefore indicate that YS110 therapy is tolerable in the clinical setting. As

described in the previous section, recent work has demonstrated the functional role of DPPIV-mediated post-translational modification of chemokines in regulating tumor immunity through its interaction with its substrate CXCL10 (101). This anti-tumor response is potentiated in combination with other anti-tumor immunotherapeutic approaches including CpG adjuvant therapy, adoptive T cell transfer therapy and checkpoint blockade therapy (anti-CTLA-4 and anti-PD-1) (101). In view of these recent findings, data from our current trial showing that serum DPPIV activity was decreased following treatment with YS110 in a dose-dependent manner would suggest that anti-tumor activity via DPPIV inhibition may constitute yet another mechanism of action for the anti-tumor activity of YS110.

4.2.3. Mechanisms of action of YS110 for cancer treatment

We previously showed that depletion of CD26 by RNAi results in the loss of adhesive property, suggesting that CD26 is a binding protein to the ECM (68). Moreover, our observations regarding the CD26-CD9-α5β1 integrin complex suggest that CD26 regulates the interaction of MPM cells with the ECM via yet-to-be-determined integrin adhesion molecules (121). Recently, we found that expression of CD26 upregulates periostin secretion by MPM cells, leading to enhanced MPM cell migratory and invasive activity (122). Periostin is a secreted cell adhesion protein of approximately 90 kDa, which shares a homology with the insect cell adhesion molecule fasciclin 1 (FAS1) (123). We also demonstrated that the cytoplasmic region of CD26 plays a crucial role in MPM tumor biology through its linkage to somatostatin receptor 4 (SSTR4) and SHP-2 protein tyrosine phosphatase in cell membrane lipid rafts, leading to cytostatic effects in MPM cells without direct association of the ECM to CD26 by anti-CD26 mAb treatment (Figure 3) (124). In view of the findings above, we propose that CD26 forms macromolecular complexes in the cell surface of MPM by connecting periostin and ECM to intracellular signaling events (125); (i) In CD26-negative MPM cells, SSTR4 mediated inhibitory signaling to suppress cell proliferation and motility. In contrast, by locking the signaling domain of SSTR4 with CD26 association, SSTR4-mediated anti-tumor effects were abrogated, leading to increased cell proliferation and motility in CD26-positive MPM cells. (ii) In addition, CD26 regulated ECM-associated tumor cell behavior in association with integrins and periostin-ECM complex. CD9 suppressed cell invasion and migration by inhibiting the formation of CD26-α5β1 integrin complex. Moreover, expression of CD26 upregulated periostin secretion by MPM cells, leading to enhanced MPM cell migratory and invasive activity. (iii) Furthermore, periostin is a secreted cell adhesion protein. The N-terminal region regulates cellular functions by binding to integrins at the plasma membrane of the cells through cell adhesion domain. The C-terminal region of the protein regulates cell–matrix organization and interaction by binding such ECM proteins, leading to increased MPM cell motility. As a result, CD26 molecule in MPM also plays a pivotal role in connecting ECM to intracellular signaling events associated with cell proliferation and motility. It is therefore conceivable that targeting CD26 may be a novel and effective therapeutic approach for MPM.

In addition to the ECM association, our *in vitro* data indicate that YS110 induces cell lysis of MPM cells via ADCC in addition to its direct anti-tumor effect via CDKI $p27^{kip1}$ accumulation (68). More recently, we evaluated the direct anti-tumor effect of YS110 against the MPM cell lines H2452 and JMN, and investigated its effects on cell cycle and on the cell cycle regulator molecules (117). YS110 suppressed the proliferation of H2452 cells by approximately 20% in 48 hours of incubation. Cell cycle analysis demonstrated that the percentage of cells in G2/M phase increased by 8.0.% on average following YS110 treatment. In addition, level of the cell cycle regulator $p21^{cip1}$ was increased and cyclin B1 was decreased after YS110 treatment. Inhibitory phosphorylation of both cdc2 (Tyr15) and cdc25C (Ser216) was elevated. Furthermore, activating phosphorylation of p38 MAPK (Thr180/Tyr182) and ERK1/2 (Thr202/Tyr204) was augmented following 24 hours of YS110 treatment. In addition, we investigated the synergistic effects of YS110 and the anti-tumor agent pemetrexed on selected MPM cell lines in both *in vitro* and *in vivo* studies. Pemetrexed rapidly induced CD26 expression on cell surface, and treatment with both YS110 and pemetrexed inhibited *in vivo* tumor growth accompanied by a synergistic reduction in the MIB-1 index (117).

We also demonstrated that treatment with YS110, which inhibited cancer cell growth, induced nuclear translocation of both cell-surface CD26 and YS110 (126, 127). In response to YS110 treatment, CD26 was translocated into the nucleus via caveolin-dependent endocytosis, and interacted with a genomic flanking region of the *POLR2A* gene, a component of RNA polymerase II. This interaction consequently led to transcriptional repression of the *POLR2A* gene, resulting in retarded cancer cell proliferation. Furthermore, impaired nuclear transport of CD26 reversed the *POLR2A* repression induced by YS110 treatment. These findings reveal that nuclear CD26 functions in the regulation of gene expression and tumor growth, and yet another novel mechanism of action of anti-CD26 mAb therapy may involve the regulation of inducible traffic of surface CD26 molecules into the cell nucleus.

4.3. Other cancers

In contrast to our robust findings regarding the role of CD26/DPPIV on MPM, the exact role of CD26/DPPIV in other cancers remains to be elucidated, partly due to its variable expression on these tumors. In general, it is strongly expressed on some cancers, while being absent or present at low levels in others (8). Furthermore, given its multiple biological functions, including its ability to associate with several key proteins and its cleavage of a number of soluble factors to regulate their function, it is likely that the CD26/DPPIV effect on tumor biology is at least partly mediated by the effect of these biological functions on specific tumor types (128).

CD26 has also been shown to be expressed on RCC (129, 130), including the cell lines Caki-1, Caki-2, ACHN, and VMRC-RCW (116). We previously showed that anti-CD26 mAb inhibition of the Caki-2 cell line was associated with G1/S cell cycle arrest, enhanced $p27^{kip1}$ expression, down regulation of cyclin-dependent kinase 2 (CDK2) and dephosphorylation of retinoblastoma substrate (Rb) (116). We also found that anti-CD26 mAb therapy attenuated Akt activity and internalized cell surface CD26 leading to decreased CD26 binding to collagen and fibronectin. Finally, we showed that anti-CD26 mAb inhibited human RCC in a mouse xenograft model (116).

Immunofluorescence analysis revealed expression of CD26/DPPIV on peripheral blood lymphocytes of patients with B chronic lymphocytic leukemia (B-CLL), but not on peripheral B cells from normal donors (131). CD26/DPPIV could also be induced in normal B cells following treatment with IL-4, indicating that expression was regulated at the level of transcription (131). CD26/DPPIV has also been shown to be a marker for aggressive T-large granular lymphocyte (T-LGL) lymphoproliferative disorder. Our work indicated that patients with CD26-positive disease were more likely to require therapies for cytopenia and infections associated with the disease than those with CD26-negative T-LGL (132). Furthermore, CD26-related signaling may be aberrant in T-LGL as compared to T-lymphocytes from normal donors (132). Disease aggressiveness is also correlated with CD26/DPPIV expression in other subsets of T-cell malignancies including T-lymphoblastic lymphoma/acute lymphoblastic leukemia (LBL/ALL), as those with CD26-positive T-LBL/ALL had a worse clinical outcome compared to patients with CD26-negative tumors (133, 134).

JKT-hCD26WT cells resulted in a greater sensitivity to doxorubicin and etoposide compared to mock transfected cells (135-138). Jurkat cells transfected with a nonfunctional DPPIV catalytic site mutant (Ser630Ala) did not show increased doxorubicin and etoposide sensitivity, suggesting that DPPIV activity is required for chemo-sensitization. A CD26 transfectant with a mutation at the ADA binding site retains DPPIV activity and had a higher level of doxorubicin sensitivity. Surface CD26 expression and DPPIV activity are associated with increased doxorubicin sensitivity and cell cycle arrest in Jurkat cells. Also, there are differences in hyperphosphorylation and inhibition of $p34^{cdc2}$ kinase activity, phosphorylation of cdc25C, and alteration in cyclin B1 expression associated with doxorubicin sensitivity in Jurkat cell lines (136). Therefore, inhibition of CD26 increases cell survival, while increased CD26 expression is associated with decreased drug resistance. The mechanism of this decreased resistance appears to be by CD26 mediated enhanced expression of topoisomerase IIα – the target for both doxorubicin and etoposide. The increased sensitivity to doxorubicin and etoposide in CD26 expressing tumors may be important in T-cell hematologic malignancies as well as other cancers. Surface expression of CD26 increases topoisomerase IIα level in the B-cell line Jiyoye and increases doxorubicin sensitivity (139). This was demonstrated by using CD26 transfection constructs in the Jiyoye B-cell lymphoma cell line as well as by target specific siRNA inhibition of CD26 in the Karpas-299 T-cell leukemia cell line. Therefore, CD26 has effects on topoisomerase IIα and doxorubicin sensitivity in both B-cell and T-cell lines. Increased CD26/DPPIV levels are associated with increased phosphorylation of p38 and its upstream regulators – MAPK kinase (MAPKK) 3/6 and apoptosis signal-regulating kinase 1 (ASK1). Therefore, the p38 signaling pathway may be involved in the regulation of topoisomerase IIα expression. Doxorubicin treated SCID mice had increased survival in those injected with wild type CD26 compared to vector or DPPIV catalytic site mutant (Ser630Ala) injected mice. CD26/DPPIV levels may be useful predictive markers for doxorubicin treatment of cancer. CD26 level is also associated with etoposide resistance. CD26 mediated changes include hyperphosphorylation of $p34^{cdc2}$, variation in cdc25C level and phosphorylation, and changes in cyclin B1 level. Since CD26/DPPIV cleaves substrates resulting in altered function (140, 141), it is possible that CD26-associated drug sensitivity may therefore be mediated by serum-derived factors. However, our work showed that the increased doxorubicin and etoposide sensitivity of JKT-hCD26WT was independent of serum, data which suggest an effect of CD26 on cell-mediated processes, such as signal transduction, rather than serum-derived factors (137).

Pang *et al.* identified a subpopulation of $CD26^+$ cells uniformly presenting in both primary and metastatic tumors in colorectal cancer (CRC), and showed that $CD26^+$ cancer cells were associated with enhanced invasiveness and chemoresistance (142). These investigators showed that in $CD26^+$ CRC cells, mediators of epithelial to mesenchymal transition (EMT) contribute to the invasive phenotype and metastatic capacity. These results suggest that $CD26^+$ cells are cancer stem cells in CRC, and that CD26/DPPIV can be targeted for metastatic CRC therapy. Recently, other investigators demonstrated in a murine model that lung metastasis of CRC was suppressed by treatment with a DPPIV inhibitor (143). They showed a reduction of EMT markers, suggesting that the EMT status of the murine colon cancer cell line MC38 was at least in part affected by DPPIV inhibition, with a diminution in the growth of metastases. They also showed that DPPIV inhibition decreased the growth of lung metastases of colon cancer by downregulating autophagy, increasing apoptosis and arresting the cell cycle. These data therefore suggest that DPPIV inhibition may be an effective therapeutic strategy for the treatment of cancers with pulmonary metastases (143).

Yamada *et al.* comprehensively investigated gene expression profiles in surgical samples of untreated gastrointestinal stromal tumors (GIST) of the stomach and small intestine. They found that the disease-free survival of patients with CD26-positive GIST of the stomach was worse than that of patients with CD26-negative GIST (144). Moreover, the postoperative recurrence rate of CD26-negative gastric GIST cases was as low as 2.0.%. They concluded that CD26 is a significant prognostic factor of gastric GIST and may also serve as a therapeutic target (144). Meanwhile, CD26 expression was not associated with clinical outcome of small intestinal GIST.

5. SUMMARY AND PERSPECTIVES

Initially described in 1966 as an enzyme with intrinsic DPPIV activity (145), this activity was subsequently found to be identical to CD26, a 110 kDa extracellular membrane-bound glycoprotein expressed on many tissues including brain, endothelium, heart, intestine, kidney, liver, lung, skeletal muscle, pancreas, placenta, and lymphocytes (26, 146, 147). Originally characterized as a T cell differentiation antigen, CD26 is preferentially expressed on a specific population of T lymphocytes, the subset of $CD4^+CD45RO^+$ memory T cells, and is upregulated following T cell activation (15, 26). Besides being a marker of T cell activation, CD26 is also associated with T cell signal transduction processes as a costimulatory molecule (5-7). CD26 therefore has an important role in T cell biology and overall immune function, and represents a novel therapeutic target for various immune disorders (13, 52, 148-150). CD26 is also expressed on various tumors such as MPM, CRC, RCC, hepatocellular carcinoma, lung cancer, prostate cancer, GIST, thyroid cancer, and hematologic malignancies such as T-anaplastic large cell lymphoma and T-LBL/ALL (10). Moreover, in several human malignancies including CRC, chronic myeloid leukemia, gastric adenocarcinoma and MPM, CD26/DPPIV expression is reported to be a marker of cancer stem cells (142, 151-155). Given the potential role of CD26 surface expression in cancer biology, YS110 therapy may also influence tumor growth through its potential effect on the cancer stem cells of selected tumors. We recently developed novel anti-human CD26 mAbs that can be used as companion diagnostic reagents suitable for immunohistochemical staining of CD26 in formalin-fixed tissue sections with reliable clarity and intensity (155). Since these mAbs display no cross-reactivity with the therapeutic humanized anti-CD26 mAb YS110, they may be suitable for assays analyzing CD26 expression during or following treatment with YS110, with important implications in the clinical setting.

Since CD26/DPPIV has a multitude of biological functions in immune system and human tumor cells, further detailed understanding of the role of this molecule in various clinical settings may lead potentially to novel therapeutic approaches.

6. ACKNOWLEDGEMENT

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Abbreviation: AA, amino acid; ADA, adenosine deaminase; ADCC, antibody-dependent cellular cytotoxicity; aGVHD, acute graft-versus-host disease; alloHSCT, allogeneic hematopoietic stem cell transplantation; AP-1, activator protein-1; APCs, antigen presenting cells; A20-luc, luciferase-transfected A20 cell; B6 WT, parental C57BL/6 mice; Cav-Ig, soluble Fc fusion proteins containing the N-terminal domain of caveolin-1; CB, cord blood; CD26KO, CD26 knockout; cGVHD, chronic graft-versus-host disease; CRC, colorectal cancer; CTL, cytotoxic T lymphocyte; CTLA-4, cytotoxic T-lymphocyte–associated antigen 4; ΔCNS-77 Tg mice, mice carrying human *IFNG* transgene with deleting *IL26* transcription; DPPIV, dipeptidyl peptidase IV; ECM, extracellular matrix; EGR2, early growth response 2; ELISA, enzyme-linked immunosorbent assay; EMT, epithelial to mesenchymal transition; ERK, extracellular signal-regulated kinase; FIH, first-in-human; GIST, gastrointestinal stromal tumors; GVHD, graft-versus-host disease; GVL, graft-versus-leukemia; HuCB, human umbilical cord blood; IBD, inflammatory bowel diseases; i.d., intradermal injection; IFN, interferon; IL, interleukin; IMQ, imiquimod; JKT-hCD26WT, Jurkat cells transfected with full-length human CD26/DPPIV; LAG3, lymphocyte activation gene-3; LBL/ALL, lymphoblastic lymphoma/acute lymphoblastic leukemia: mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; MERS-CoV, Middle East respiratory syndrome coronavirus; MERS-CoV S1-Fc, S1 domain of MERS-CoV fused to the Fc region of human IgG; MPM, malignant pleural mesothelioma; NFAT, nuclear factor of activated T cells; NOG, NOD/Shi-*scid*IL2rγ^{null}; OB, obliterative bronchiolitis; PBL, peripheral blood lymphocyte; PD-1, programmed cell death 1; PSO, psoriasis; RA, rheumatoid arthritis; RBD, receptor binding domain; RCC, renal cell carcinoma; RECIST, response evaluation criteria in solid tumors; sCD26, soluble CD26; sDPPIV, soluble dipeptidyl peptidase IV; siRNA, small interfering RNA; SP, substance P; SSTR4, somatostatin receptor 4; Tg, transgenic; T-LGL, T-large granular lymphocyte; TME, tumor microenvironment; TNF, tumor necrosis factor; Tr1, Type 1 regulatory T cells; WHO, World Health Organization; 190-*IFNG* Tg mice, mice carrying human *IFNG* and *IL26* transgene

Key Words CD26, DPPIV, Caveolin-1, Humanized anti-CD26 monoclonal antibody, Graft-versus-host disease, Malignant pleural mesothelioma, Review

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Figure 1. Schematic diagram of human CD26 profiling the predicted contacting areas of anti-CD26 mAbs 2F9, 1F7, YS110 and MERS-CoV S1. 2F9 recognizes between 248-449th AAs including the ADA binding regions, and 1F7 or YS110 recognize between 248-358th AAs excluding the ADA binding regions. MERS-CoV contacting residues of human CD26 are indicated in stars, with available information obtained from recently published data (55, 69). TM indicates the transmembrane region of human CD26 (black box), and the extracellular domain of CD26 is located at the C-terminal residues of TM. This Figure is reprinted with permission from K Ohnuma *et al.*, *J Virol* 87: 13892-13899, 2013 (62).

Figure 2. DPP4 inhibition suppresses truncation of its ligand CXCL10, leading to recruitment of $CXCR3^+$ T cells into tumor parenchyma. Through an *in vivo* xenotransplant models, DPPIV inhibitor is found to reduce tumor growth through the preservation of bioactive CXCL10 in the tumor microenvironment (TME). In the normal physiological state, CXCL10 is rapidly degraded by CD26/DPPIV, resulting in decreased recruitment and migration of $CXCR3^+$ T cells into the tumor parenchyma. In contrast, DPPIV inhibition enhances tumor rejection by preserving the full-length biologically active form of CXCL10, leading to increased trafficking of $CXCR3^+$ T cells into the tumor parenchyma. This anti-tumor response is potentiated in combination with other anti-tumor immunotherapeutic approaches including CpG adjuvant therapy, adoptive T cell transfer therapy and checkpoint blockade therapy. This Figure is reprinted with permission from K Ohnuma *et al.*, *Nat Immunol* 16: 791-792, 2015 (156).

Figure 3. A model for cytostatic signaling mediated by SSTR4 and CD26 coassociation in MPM cells. SSTR4 molecules form homo- or oligo-dimers when stimulated by its agonists (Ago), followed by manifestation of their cytostatic effects via SHP-2 signaling **(A)**. When coassociation of SSTR4 with CD26 occurs, CD26 binds to the C-terminal region of SSTR4, which is necessary to transduce SSTR4 signaling, hence blocking the SSTR4-mediated cytostatic effects **(B)**. Meanwhile, anti-CD26 mAb ligates CD26, leading to dissociation of SSTR4 from CD26 and to recruiting lipid rafts with clustering of SSTR4 molecules **(C)**. As a result, downstream signaling of SSTR4 occurs with activation of SHP-2, leading to the observed cytostatic effects.

Running title: CD26 as therapeutic target

Figure 1

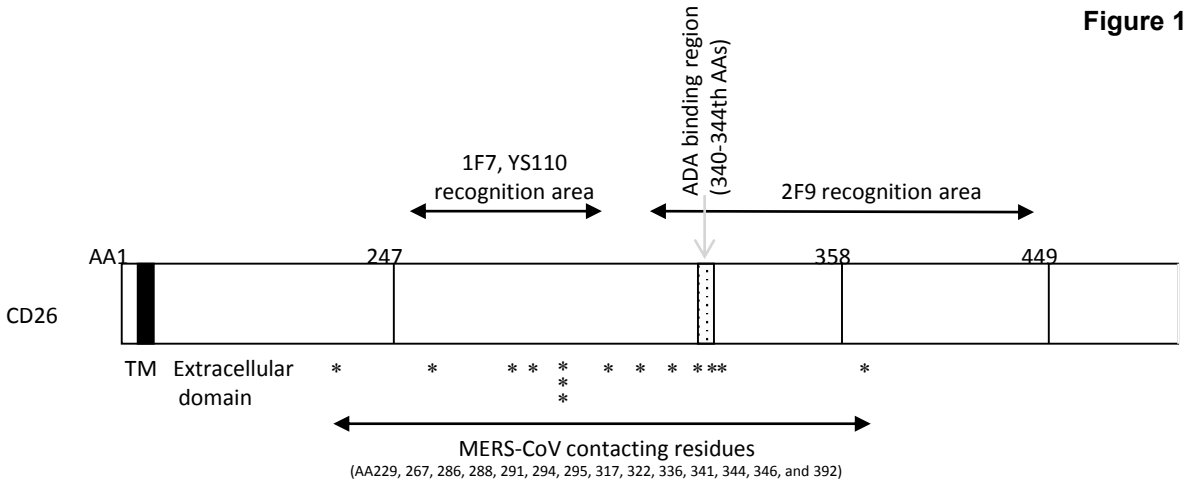


Figure 2

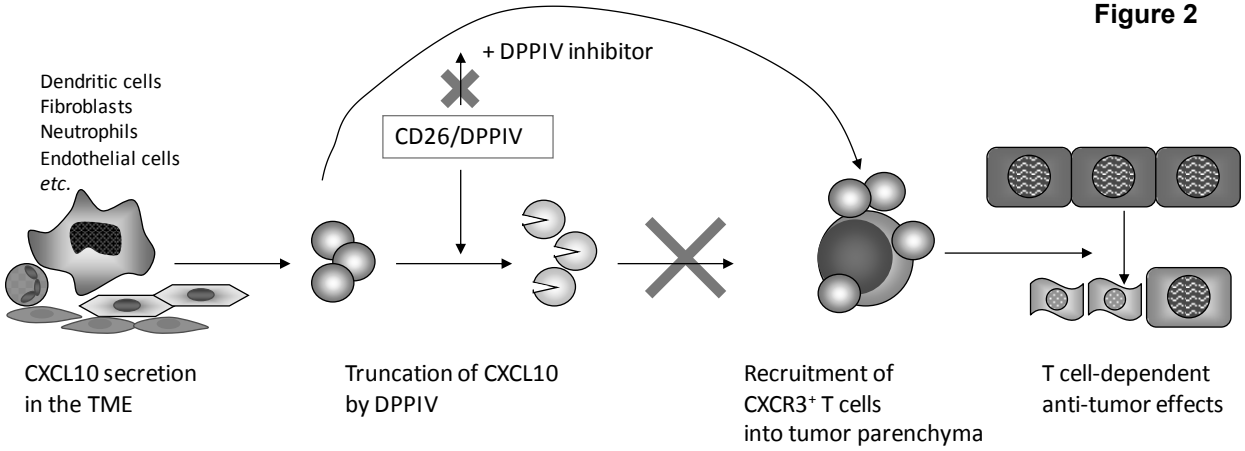
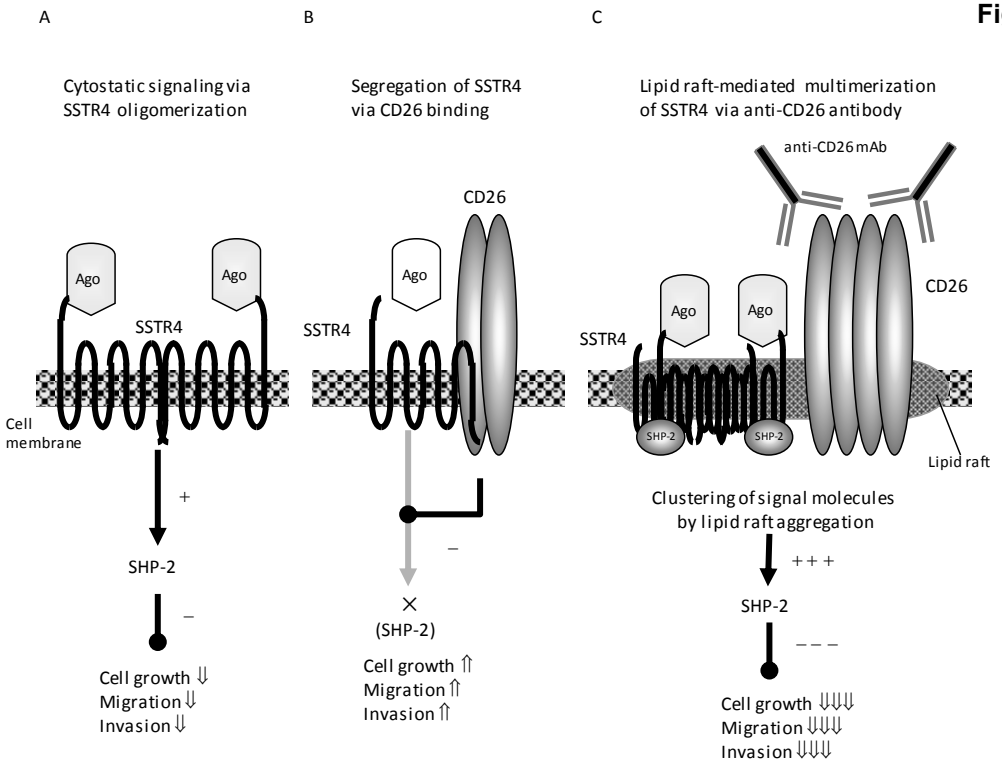


Figure 3



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Caveolin-1, a binding protein of CD26, is essential for the anti-inflammatory effects of dipeptidyl peptidase-4 inhibitors on human and mouse macrophages

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ABSTRACT

We previously reported that inhibition of dipeptidyl peptidase (DPP)-4, the catalytic site of CD26, prevents atherosclerosis in animal models through suppression of inflammation; however, the underlying molecular mechanisms have not been fully elucidated. Caveolin-1 (Cav-1), a major structural protein of caveolae located on the surface of the cellular membrane, has been reported to modulate inflammatory responses by binding to CD26 in T cells. In this study, we investigated the role of Cav-1 in the suppression of inflammation mediated by the DPP-4 inhibitor, teneligliptin, using mouse and human macrophages. Mouse peritoneal macrophages were isolated from Cav-$1^{+/+}$ and Cav-$1^{-/-}$ mice after stimulation with 3% thioglycolate. Inflammation was induced by the toll-like receptor (TLR)4 agonist, lipopolysaccharide (LPS), isolated from *Escherichia coli*. The expression of pro-inflammatory cytokines was determined using reverse transcription-polymerase chain reaction. Co-expression of Cav-1 and CD26 was detected using immunohistochemistry in both mouse and human macrophages. Teneligliptin treatment (10 nmol/L) suppressed the LPS-induced expression of interleukin (IL)-6 (70%) and tumor necrosis factor-α (37%) in peritoneal macrophages isolated from Cav-$1^{+/+}$ mice. However, teneligliptin did not have any effect on the macrophages from Cav-$1^{-/-}$ mice. In human monocyte/macrophage U937 cells, teneligliptin treatment suppressed LPS-induced expression of pro-inflammatory cytokines in a dose-dependent manner (1−10 nmol/L). These anti-inflammatory effects of teneligliptin were mimicked by gene knockdown of Cav-1 or CD26 using small interfering RNA transfection. Furthermore, neutralization of these molecules using an antibody against CD26 or Cav-1 also showed similar suppression. Teneligliptin treatment specifically inhibited TLR4 and TLR5 agonist-mediated inflammatory responses, and suppressed LPS-induced phosphorylation of IL-1 receptor-associated kinase 4, a downstream molecule of TLR4. Next, we determined whether teneligliptin could directly inhibit the physical interaction between Cav-1 and CD26 using the Biacore system. Binding of CD26 to Cav-1 protein was detected. Unexpectedly, teneligliptin also bound to Cav-1, but did not interfere with CD26-Cav-1 binding, suggesting that teneligliptin competes

Abbreviations: ANOVA, analysis of variance; Cav-1, caveolin-1; DAPI, 4′, 6-diamidino-2-phenylindole; DPP-4, dipeptidyl peptidase-4; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GST, glutathione *S*-transferase; IL, interleukin; IP, interferon gamma-induced protein; IRAK, IL-1 receptor-associated kinase; LPS, lipopolysaccharide; MCP, monocyte chemoattractant protein; MEM, minimum essential medium; NF-κβ, nuclear factor-kappa beta; PBS, phosphate buffered saline; PE, phycoerythrin; rhCD26, recombinant human CD26; RT-PCR, reverse transcription-polymerase chain reaction; RU, response unit; SEM, standard error of mean; siRNA, small interfering RNA; SDS, sodium dodecyl sulfate; TLR, toll-like receptor; TNF, tumor necrosis factor.

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with CD26 for binding to Cav-1. In conclusion, we demonstrated that Cav-1 is a target molecule for DPP-4 inhibitors in the suppression of TLR4-mediated inflammation in mouse and human macrophages.

1. Introduction

Dipeptidyl peptidase (DPP)-4 inhibitors are commonly used for the treatment of patients with type 2 diabetes because of their potent glucose-lowering effect, without any increase in hypoglycemia risk and body weight gain [1–3]. DPP-4, originally known as the catalytic site of the T-cell surface marker CD26, is widely expressed in most cells [4]. DPP-4 cleaves incretin hormones, such as glucose-dependent insulinotropic polypeptide and glucagon-like peptide-1 that stimulate insulin secretion in pancreatic β-cells. In addition, various peptides, which are involved in the regulation of immune and cardiovascular systems, are also cleaved by DPP-4 [5,6]. Extensive studies have been conducted to evaluate the direct vascular protective and anti-inflammatory effects of DPP-4 inhibitors. We previously reported that the DPP-4 inhibitor, vildagliptin, suppresses the progression of atherosclerosis in diabetic apolipoprotein E-null mice, and this effect is partially incretin-independent [7]. Furthermore, the direct anti-atherogenic effects of DPP-4 inhibitors on vascular cells and macrophages have been shown in vitro [7–14]. However, the underlying molecular mechanisms are not fully elucidated.

Caveolin-1 (Cav-1), a major structural protein of caveolae on the cell surface, is implicated in regulating inflammatory responses [15]. Ohnuma et al. have demonstrated an interaction between Cav-1 and CD26 in antigen-presenting cells, including T cells [16–19]. CD26 phosphorylates Cav-1 by direct binding, resulting in the activation of the nuclear factor-kappa beta (NF-κβ) pathway [17]. In addition, the enzymatic activity of CD26 is essential for its interaction with Cav-1 [19]. However, the relationship between CD26 and Cav-1 in other immune cells and the involvement of Cav-1 in the anti-inflammatory effects of DPP-4 inhibitors are yet to be determined. In the present study, we demonstrated that Cav-1 is an essential molecule for the suppression of toll-like receptor (TLR)4-induced inflammation mediated by DPP-4 inhibitors in mouse and human macrophages.

2. Materials and methods

2.1. Animal study

This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (8th edition, 2011; Office of Laboratory Animal Welfare, National Institutes of Health, MD, USA). All procedures were approved by the Animal Care Committee of Showa University School of Medicine (approval number 06013). Cav-1 knockout (Cav-$1^{-/-}$) mice and their littermate controls (Cav-$1^{+/+}$) were purchased from the Jackson Laboratory (ME, USA), and kept on standard rodent chow. Six–eight-week-old female mice were intraperitoneally injected with 4 mL of 3% thioglycolate (Wako, Osaka, Japan). Four days after injection, mice were euthanized by anesthesia overdose using isoflurane. Exudate peritoneal macrophages were collected by injecting ice-cold phosphate buffered saline (PBS) into the intraperitoneal cavity. The collected cells were seeded into 3.5-cm dishes (1×10^6 cells/dish) and allowed adhesion to the dish for 1 h. The adherent cells were identified as peritoneal macrophages [20]. The cells were treated with lipopolysaccharide (LPS) (B4; Sigma-Aldrich, MO, USA) with or without the DPP-4 inhibitor (teneligliptin; gifted from Mitsubishi Tanabe Pharma, Osaka, Japan) in RPMI 1640 medium (Gibco, MA, USA) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 U/mL streptomycin, and 1% minimum essential medium (MEM) non-essential amino acid solution.

2.2. Cell culture

A human monocyte/macrophage cell line, U937, was obtained from JCRB (JCRB9021; Osaka, Japan). The cells were cultured in RPMI 1640 medium containing 10% FBS. The cells were seeded into 3.5-cm dishes (1×10^6 cells/dish) and treated with LPS with or without reagents for the indicated hours. The following antibodies were used for the neutralization of target proteins: anti-CD26 antibody (H-270; sc-9153; Santa Cruz Biotechnology, TX, USA; RRID: AB_2093585), anti-Cav-1 antibody (H97; sc-7875; Santa Cruz Biotechnology; RRID: AB_2072020), and anti-Cav-1 antibody (7c8; sc-53564; Santa Cruz Biotechnology; RRID: AB_628859). TLR agonists that were used are presented in Supplemental Table 1.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) and used to synthesize complementary DNA using ReverTra Ace® (TOYOBO, Osaka, Japan). Real-time RT-PCR was performed using TaqMan gene expression assays and ABI 7900HT (Applied Biosystems, MA, USA). The following probe sets were used: interleukin-1β (*Il-1β*), Hs01555410_m1; *Il-6*, Hs00985639_m1 and Mm00446190_m1; tumor necrosis factor-α (*Tnf-α*), Hs01113624_g1 and Mm00443258_m1; *Nf-κβ*, Hs00765730_m1; interferon gamma-induced protein 10 (*Ip-10*), Hs00171042_m1; monocyte chemoattractant protein-1 (*Mcp-1*), Hs00234140_m1; 18S ribosomal RNA (*18s rRNA*), Hs999999901_s1; and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), Mm03302249_g1. Expression of target gene was normalized to that of an internal control (*18s rRNA*) in U937 cells or *Gapdh* in mouse peritoneal macrophages.

2.4. Biophysical interaction analysis

In vitro protein binding was assessed using Biacore™ T100 (GE Healthcare, Buckinghamshire, England). The buffer consisted of 0.1 mol/L HEPES, 1.5 mol/L NaCl, 30 mmol/L EDTA, and 0.5% (v/v) surfactant P20. Glutathione *S*-transferase (GST)-tagged Cav-1 (Cav-1 human recombinant protein; Abnova, Taipei, Taiwan) at 5 μg/mL was immobilized on Sensor Chip CM5 (GE Healthcare) as a ligand using GST capture kit (GE Healthcare). Recombinant human CD26 (rhCD26; R&D Systems, MN, USA; 25 μg/mL), teneligliptin (100 μmol/L or 1 mmol/L), or rhCD26 (25 μg/mL) + teneligliptin (100 μmol/L or 1 mmol/L) were injected onto the sensor chip as analytes for 150 s. Glycine-HCL (10 mmol/L) was used to regenerate the immobilized sensor chip surface. Mass change on the sensor tip induced by binding between molecules was detected as response unit (RU).

2.5. Immunohistochemistry

Mouse peritoneal macrophages and U937 cells were seeded in poly-D-lysine-coated microplates and fixed with acetone-methanol (1:1) for 2 min at 23 °C. After blocking with 5% bovine serum albumin-PBS for 30 min at 4 °C, the fixed cells were incubated with primary antibodies for 1 h at 37 °C and secondary antibodies for 1 h at 37 °C. The primary antibody used for U937 cells was anti-CD26 antibody (generated by Ohnuma; 5 μg/mL; raised in rabbit) or anti-Cav-1 antibody (sc-894; Santa Cruz Biotechnology; 5 μg/mL; raised in rabbit; RRID: AB_2072042). Anti-CD26 antibody (AF954; R&D Systems; 5 μg/mL; RRID: AB_355739) or anti-Cav-1 antibody (3238; Cell Signaling Technology, MA, USA; 5 μg/mL; RRID: AB_10699017) was used for mouse peritoneal macrophages. The cells were mounted with ProLong™ Gold Antifade (Thermo Fisher Scientific, MA, USA), and images were taken with the BZ8000 microscope (KEYENCE, Osaka, Japan).

2.6. Immunoblotting

U937 cells were treated with various reagent concentrations for the indicated time periods. After incubation, the cells were washed with PBS three times and lysed using 10% sodium dodecyl sulfate (SDS). The lysates were used for protein measurement by bicinchoninic acid (Thermo Fisher Scientific), and boiled for 5 min in NuPAGE LDS Sample Buffer (3:1; Thermo Fisher Scientific). The samples (10 μg/lane) were subjected to 10% SDS-polyacrylamide gel electrophoresis, and transferred to Immobilon-P membranes (Merck Millipore, Darmstadt, Germany). The membranes were immunoblotted with antibodies against IL-1 receptor-associated kinase 4 (IRAK-4) (2H9; MAB10701; Abnova, Taipei, Taiwan; 1:500; raised in rabbit; RRID: AB_11190488), phosphorylated IRAK-4 (p-IRAK4; T345) (A8A8; MAB2538; Abnova; 1:500; raised in rabbit; RRID: AB_10555313), and β-actin (C4; sc47778; Santa Cruz Biotechnology, TX, USA; 1:200; raised in rabbit; RRID: AB_626632). The protein bands were visualized with enhanced luminal reagents (PerkinElmer, MA, USA) and quantified using FIJI software (NIH, MD, USA).

2.7. Small interfering RNA transfection

Small interfering (si)RNAs against control (sc-37007), Cav-1 (sc-29241), and CD26 (sc-42762) were obtained from Santa Cruz Biotechnology (TX, USA). Transfection was performed using X-tremeGENE siRNA transfection reagent (Roche, Basel, Switzerland) with 25 nmol/L of siRNA. The cells were used for experiments 48 h post transfection.

2.8. Cell viability

Cell viability was evaluated using CellQuanti-MTT Cell Viability Assay Kits (BioAssay Systems, CA, USA). U937 cells were seeded into 96-well plates (1×10^4 cells/well) and treated with the indicated concentrations of reagents for 22 h in 5% FBS-RPMI 1640 medium. During the last 4 h, the cells were incubated with CellQuanti-MTT reagent. Solubilization solution was added to each well, and absorbance at 570 nm was measured using infinite M200 PRO (Tecan, Männedorf, Switzerland).

2.9. Flow cytometry

Mouse peritoneal macrophages were treated with or without LPS (100 ng/mL) or teneligliptin (10 μmol/L) for 1 h, and stained with phycoerythrin (PE)-conjugated anti-CD11b antibody (Bio-Legend, CA, USA) and fluorescein isothiocyanate (FITC)-conjugated anti-CD26 antibody (Bio-Rad Laboratories, CA, USA). Fluorescence intensity was measured using BD FACSVerse™ (Becton, Dickinson and Company, NJ, USA), and data obtained were analyzed by FlowJO software (Tree Star, Inc., OR, USA).

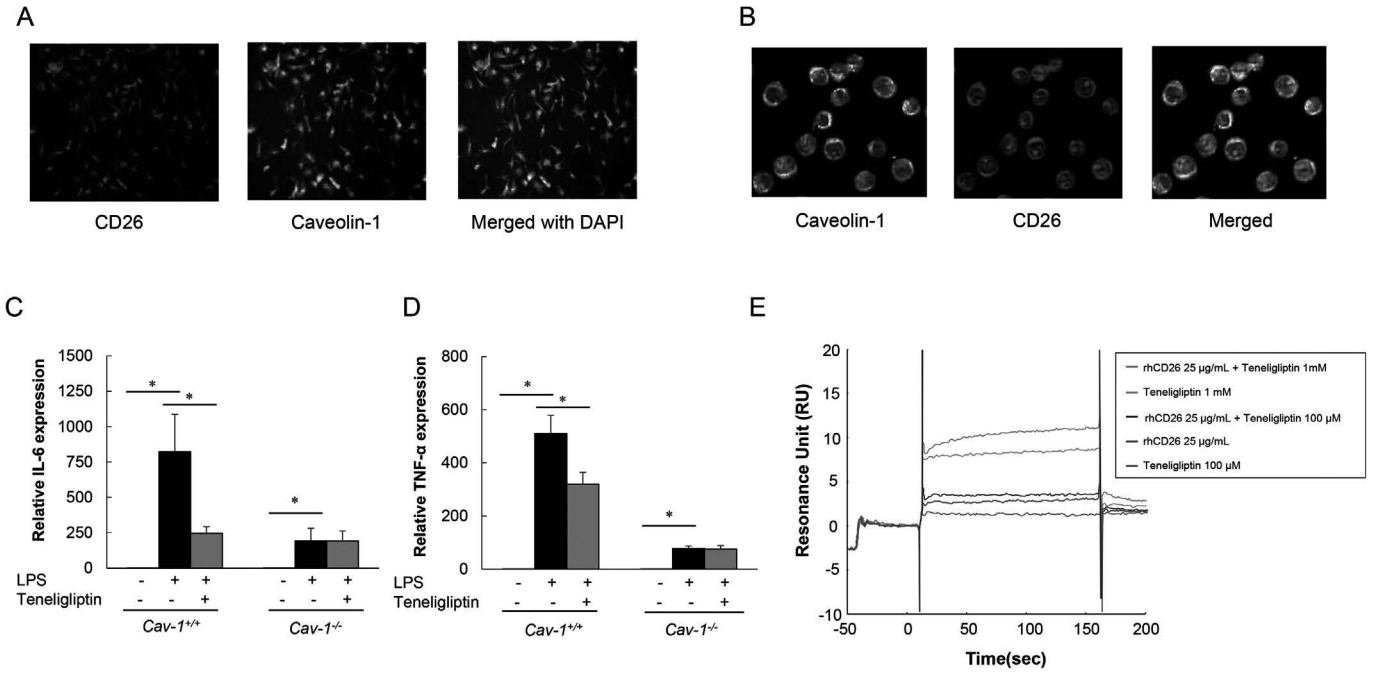

Fig. 1. The role of Cav-1 in the anti-inflammatory effects of teneligliptin. (A and B) Expression of CD26 and Cav-1 in mouse peritoneal macrophages and human U937 cells. Representative images of immunofluorescent staining of CD26 and Cav-1, and the merged images with 4′,6-diamidino-2-phenylindole (DAPI) are shown in A (mouse peritoneal macrophages) and B (human U937 cells). **(C and D)** The effects of teneligliptin on LPS-induced expression of *Il-6* and *Tnf-α* in peritoneal macrophages isolated from Cav-$1^{+/+}$ and Cav-$1^{-/-}$ mice. n = 4–6 per group. *$p < 0.05$. **(E)** Binding of rhCD26 and teneligliptin to Cav-1 protein was determined using Biacore system. The binding of protein is expressed as RU.

2.10. Statistical analysis

Values are expressed as the means ± standard error of mean (SEM). Statistical comparisons were performed by analysis of variance (ANOVA) followed by Tukey's test using JMP software (version 11; SAS Institute Inc., NC, USA). The significance level was defined as $p < 0.05$.

3. Results

3.1. Co-expression of CD26 and Cav-1 in mouse peritoneal macrophages and human U937 cells

First, we investigated whether CD26 and Cav-1 are expressed in mouse peritoneal macrophages or U937 cells. Immunofluorescent staining showed that CD26 and Cav-1 were co-expressed primarily in the cytoplasm of mouse peritoneal macrophages and U937 cells (Fig. 1A and B). In flow cytometric analysis, less than 10% of the mouse peritoneal macrophages were positive for CD26. LPS (100 ng/mL) and teneligliptin (10 μmol/L) did not affect the ratio of CD26-positive cells (Supplemental Fig. 1).

3.2. Cav-1 is involved in the anti-inflammatory effects of teneligliptin on mouse peritoneal macrophages

We evaluated whether Cav-1 is involved in the anti-inflammatory effects of teneligliptin using mouse peritoneal macrophages isolated from Cav-$1^{+/+}$ or Cav-$1^{-/-}$ mice. Treatment with LPS (100 ng/mL for 4 h), a TLR4 agonist, significantly increased *Il-6* and *Tnf-α* expression in the macrophages from Cav-$1^{+/+}$ mice, while co-treatment with teneligliptin (10 nmol/L) suppressed their expression by 70 and 37%, respectively. In Cav-$1^{-/-}$ mice, LPS treatment also increased the expression of *Il*-6 and *Tnf-α*. However, responses to LPS stimulation were lower than those observed in Cav-$1^{+/+}$ mice. Unlike that in Cav-$1^{+/+}$ mice, teneligliptin failed to suppress LPS induced expression of *Il*-6 and *Tnf-α* in Cav-$1^{-/-}$ mice (Fig. 1C and D).

3.3. Teneligliptin does not affect protein binding of CD26 and Cav-1 in vitro

A previous study using biophysical interaction analysis has shown that CD26 protein directly binds to Cav-1 protein in vitro [19]. We determined whether teneligliptin affects the interaction between CD26 and Cav-1 protein using the Biacore system, which is capable of assessing direct molecule binding by measuring mass change on the sensor chip. In this analysis, the binding of the protein was expressed as RU. Cav-1 protein was immobilized on the sensor chip as a ligand, and rhCD26 and teneligliptin were used as analytes. Administration of rhCD26 to Cav-1 protein showed elevated RU levels compared to Cav-1 protein alone. Furthermore, administration of teneligliptin (100 μmol/L or 1 mmo/L) to Cav-1 protein dose-dependently increased RU levels, indicating the direct binding of teneligliptin to Cav-1. However, co-administration of teneligliptin and rhCD26 showed higher RU levels than those observed with administration of rhCD26 or teneligliptin alone (Fig. 1 E).

3.4. Teneligliptin suppresses LPS-induced expression of pro-inflammatory molecules in U937 cells

Next, we evaluated the effects of teneligliptin on the expression

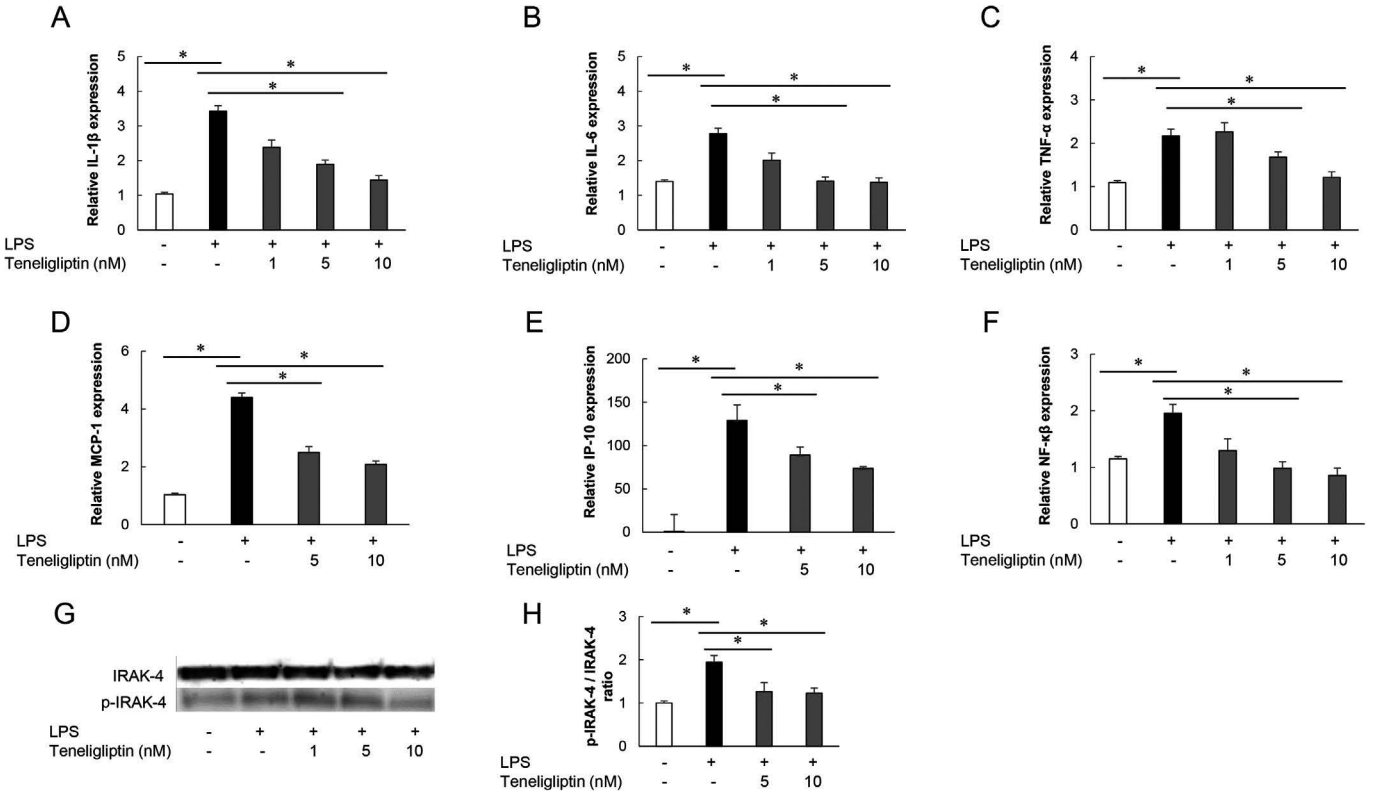

Fig. 2. The anti-inflammatory effects of teneligliptin on human macrophages. (A–F) The effect of teneligliptin on the expression of pro-inflammatory molecules in human U937 cells. Expression of *Il-1β*, *Il-6*, *Tnf-α*, *Mcp-1*, *Ip-10*, and *Nf-κβ* was measured by real-time RT-PCR. **(G)** and **(H)** The effect of teneligliptin on LPS-mediated phosphorylation of IRAK-4 in U937 cells. Representative images of immunoblot bands for phosphorylated and total IRAK-4 **(G)**. The ratio of phosphorylated IRAK-4 to total IRAK-4 **(H)**. n = 3 per group. *$p < 0.05$.

of pro-inflammatory molecules in human U937 cells. LPS treatment (10 μg/mL) increased the expression of *Il-1β*, *Il-6*, and *Tnf-α* at 6 h, and co-treatment with teneligliptin (1, 5, and 10 nmol/L) dose-dependently suppressed this increase (Fig. 2 A–C). Furthermore, teneligliptin treatment at 5 and 10 nmol/L also suppressed LPS-induced expression of *Mcp-1*, *Ip-10*, and *Nf-κβ* (Fig. 2 D–F). Consistently, teneligliptin treatment (5 and 10 nmol/L) suppressed LPS-induced phosphorylation of IRAK-4, a downstream molecule of TLR4 signaling (Fig. 2 G); however, it did not affect cell viability in the presence or absence of LPS (Supplemental Fig. 2).

Neutralization and gene knockdown of CD26 or Cav-1 suppress LPS-induced expression of pro-inflammatory molecules in U937 cells.

To obtain further insights into the role of CD26 and Cav-1 in TLR4-induced inflammatory responses, we conducted antibody neutralization and siRNA-induced gene knockdown against CD26 or Cav-1. Anti-CD26 antibody (100 ng/mL) suppressed LPS-induced expression of *Il-1β*, *Il-6*, and *Tnf-α* to the same level as that achieved by teneligliptin treatment (Fig. 3 A–C). Similarly, anti-Cav-1 polyclonal antibody (H97; 200 ng/mL), recognizing amino acids 82–178 of Cav-1, significantly suppressed LPS-induced expression of these cytokines. In contrast, anti-Cav-1 monoclonal antibody (7c8; 200 ng/mL), recognizing amino acids between residue 32 and the C-terminus of Cav-1, did not show any effect (Fig. 3 D–F). Expression of CD26 or Cav-1 was significantly decreased by siRNA transfection (Supplemental Fig. 3). Gene knockdown of CD26 and Cav-1 significantly attenuated LPS-induced expression of *Il-1β*, *Il-6*, or *Tnf-α* (Fig. 3 G–I).

3.5. The anti-inflammatory effects of teneligliptin are specific to TLR4 and TLR5 signaling pathways

Finally, we evaluated the effects of teneligliptin on inflammation induced by other TLR agonists. U937 cells were stimulated with specific agonists for TLR1–9. All agonists significantly increased the expression of *Il-1β*, *Il-6*, and *Tnf-α* (Fig. 4 A–C). Teneligliptin (5 nmol/L) suppressed the expression of these pro-inflammatory cytokines induced by LPS (TLR4 agonist) and flagellin (TLR5 agonist), but did not suppress the expression of cytokines induced by other TLR agonists.

4. Discussion

In the present study, we demonstrated that Cav-1 is a target of DPP-4 inhibitors in the suppression of inflammation in human and murine macrophages. Teneligliptin is a potent DPP-4 inhibitor that inactivates DPP-4 at a lower IC50 (0.37 nmol/L) than other DPP-4 inhibitors (IC50, 1–62 nmol/L) [21], and is wildly used for the treatment of type 2 diabetes. The anti-inflammatory effects of DPP-4 inhibitors have been shown in various immune cells of rodents and humans [8,13,14]. Consistently, teneligliptin suppressed TLR4 ligand (LPS)-induced inflammation in mouse exudate peritoneal macrophages and human U937 cells, both of which co-expressed CD26 and Cav-1. It is noteworthy that the genetic deletion of Cav-1 in macrophages reduced inflammatory responses to LPS derived from *Escherichia coli*, and that teneligliptin treatment did not suppress LPS-induced inflammation in macrophages in the absence of Cav-1.

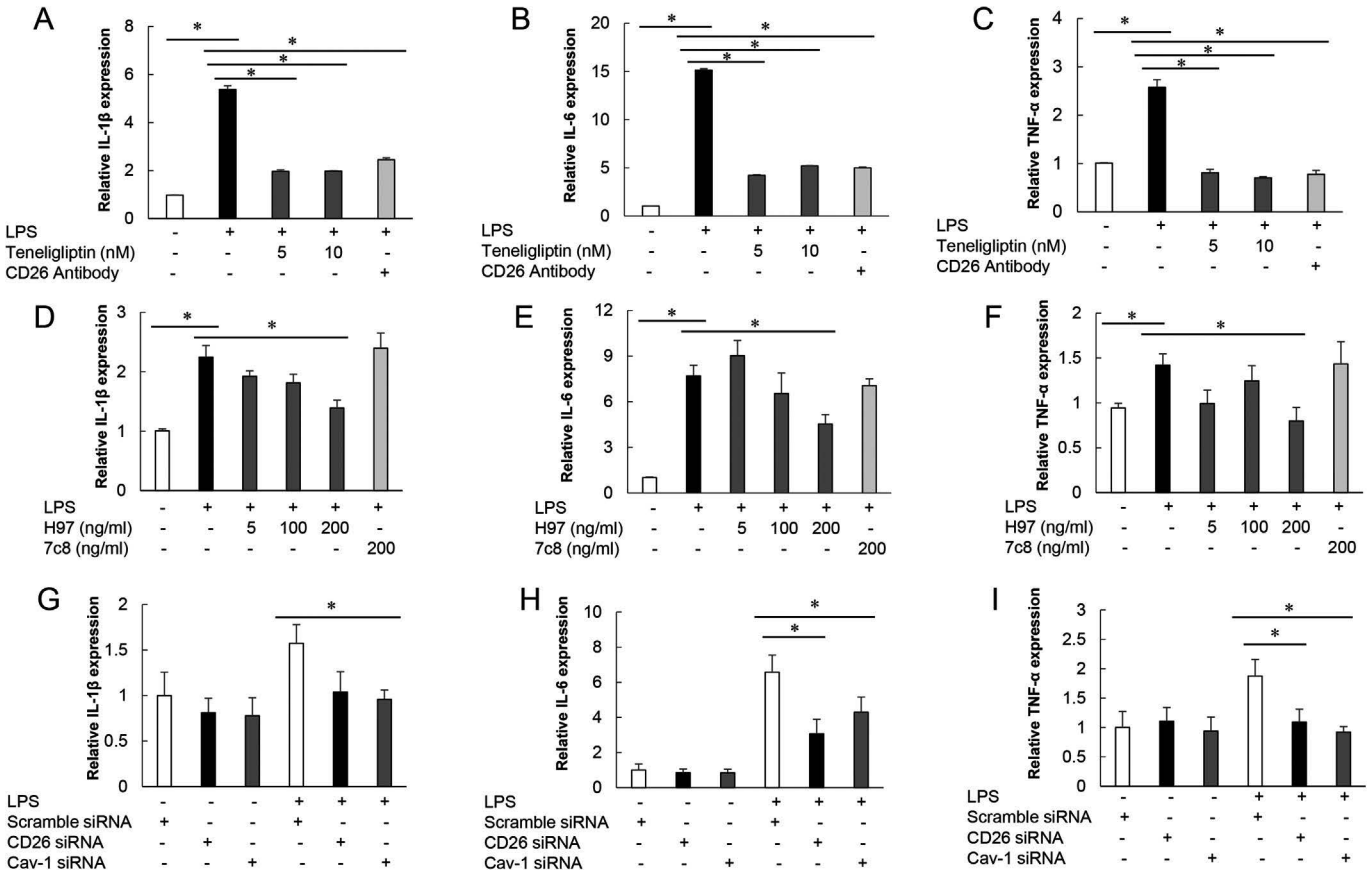

Fig. 3. The role of CD26 and Cav-1 on TLR4-induced inflammation. (A–F) The effect of neutralization by anti-CD26 or anti-Cav-1 antibody (polyclonal H97 or monoclonal 7c8) on LPS-induced expression of pro-inflammatory cytokines in U937 cells. Relative expression of *Il-1β*, *Il-6*, and *Tnf-α* to that of *18s rRNA* using anti-CD26 or anti-Cav-1 antibody is presented in A–C and D–F, respectively. n = 3–5 per group. **(G–I)** The effect of gene knockdown of CD26 or Cav-1 by siRNA transfection on LPS-induced expression of pro-inflammatory cytokines in U937 cells. n = 3–5 per group. $^{*}p < 0.05$.

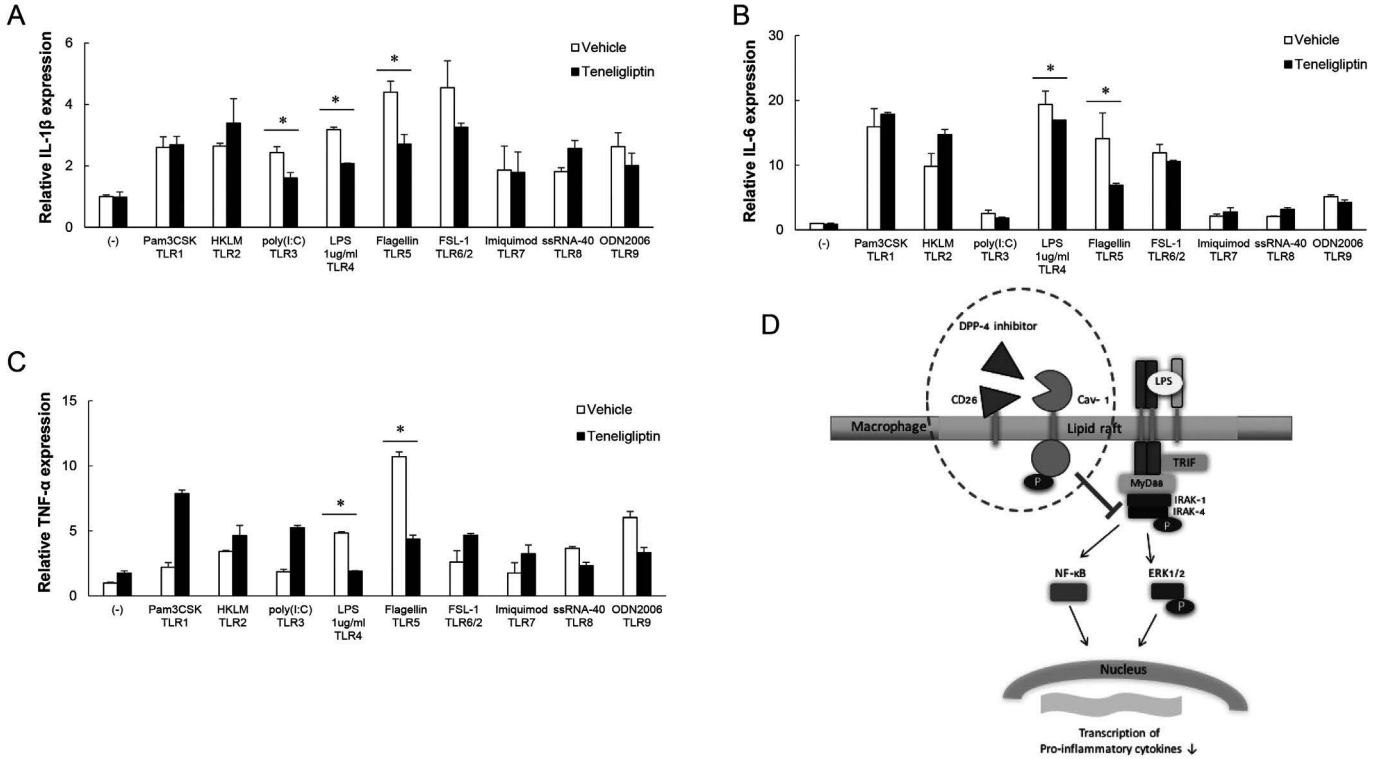

Fig. 4. TLR agonists involved in the anti-inflammatory effects of teneligliptin. (A–C) Effect of teneligliptin on the expression of pro-inflammatory cytokines (A, *Il-1β*; B, *Il-6*; C, *Tnf-α*) in U937 cells stimulated by various ligands of TLRs. The ligands of TLRs used are shown in Supplemental Table 1. n = 3 per group. *p < 0.05. **(D)** A scheme for the proposed mechanism of anti-inflammatory action of DPP-4 inhibitors in macrophages. DPP-4 inhibitors regulate the function of Cav-1 by affecting CD26 and Cav-1 interaction, resulting in the suppression of inflammatory responses via inhibiting the TLR4/IRAK-4 pathway. TRIF, TIR-domain-containing adapter-inducing interferon-β; ERK, extracellular signal-regulated kinase.

Previous studies investigating the role of Cav-1 in inflammatory responses have shown confounding results [22,23]. Cav-$1^{-/-}$ mice have been shown to be resistant to lung inflammation induced by intraperitoneal administration of LPS derived from *E. coli* via decreased NF-κβ activity [22]. In contrast, peritoneal macrophages obtained from Cav-$1^{-/-}$ mice show enhanced inflammatory responses to LPS derived from *Salmonella enterica* [23]. There is a report showing that inflammatory responses are different between LPS derived from *E. coli* and *S. enterica* and that lipid A, one of the structural components of LPS, is involved in this difference [24]. Thus, whether Cav-1 enhances or suppresses inflammatory responses may vary in different types of LPS or cells. Our data show that, in mouse macrophages, Cav-1 acts as a pro-inflammatory molecule in response to *E. coli*-derived LPS, and is a target of DPP-4 inhibitors to suppress inflammation induced by this type of LPS.

To gain further understanding about the interaction between CD26 and Cav-1, we conducted antibody neutralization and gene knockdown against CD26 and Cav-1. In human U937 cells, treatment with antibody against CD26 and siRNA transfection against Cav-1 or CD26 showed suppression in LPS-induced expression of pro-inflammatory cytokines, similar to that observed after teneligliptin treatment. However, two antibodies against Cav-1 exerted different effects: the antibody recognizing amino acids 82–178 of Cav-1 suppressed inflammatory responses to LPS, and the antibody recognizing the amino acids between residue 32 and the C-terminus of Cav-1 did not show any effect. Ohnuma et al. have demonstrated that residue 630 of CD26 (serine catalytic site having DPP-4 activity) is required to bind to Cav-1 residues 82–101 (also known as the caveolin scaffolding domain) [16]. Our findings provide further evidence that residues 82–178 of Cav-1 are essential for the inflammatory responses induced by TLR4, possibly due to their involvement in the binding of Cav-1 and CD26.

We found that teneligliptin suppress LPS-induced phosphorylation of IRAK-4 in human U937 cells. IRAK-4 has been shown to be a signaling component that is required for NF-κB activation, and is highly co-localized with Cav-1 in the caveolae of macrophages [25]. A previous study has demonstrated that Cav-1 deficiency reduces inflammatory responses to LPS by impairing IRAK-4 activity in the mouse lung [26]. Our findings indicate that suppression of IRAK-4 phosphorylation may be one of the mechanisms responsible for the anti-inflammatory effects of teneligliptin.

In conclusion, Cav-1 acts as a pro-inflammatory molecule in response to *E. coli*-derived LPS in mouse and human macrophages, and is a target of DPP-4 inhibitors to suppress TLR4-induced inflammation.

Author contribution

MH conducted in vivo and in vitro experiments, analyzed the data, and wrote the manuscript. KN conducted in vitro experiments, analyzed the data and revised the manuscript. YM analyzed the data and contributed to data interpretation and manuscript revision. HK and MS conducted in vitro experiments and contributed to data interpretation and manuscript revision. KO conducted in vitro experiments, analyzed the data, and revised the manuscript. HK contributed to data interpretation and manuscript revision. TH designed the study, and revised and finalized the manuscript. All the authors approved the final version of the manuscript for the submission.

Conflicts of interest

TH received lecture fees from MSD KK, Novartis Pharma KK,

Novo Nordisk Pharma Ltd., Sanwa Kagaku Kenkyusho Co. Ltd., Kowa Co. Ltd., Eli Lilly Japan KK, Mitsubishi Tanabe Pharma Co., and Ono Pharmaceutical Co. Ltd. All other authors declare that they have no competing interests.

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Transparency document

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bbrc.2017.11.016.

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FULL PAPER

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Keywords: CD26; phase I; mesothelioma; immune checkpoint

First-in-human phase 1 of YS110, a monoclonal antibody directed against CD26 in advanced CD26-expressing cancers

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Background: YS110 is a humanised IgG1 monoclonal antibody with high affinity to the CD26 antigen. YS110 demonstrated preclinical anti-tumour effects without significant side effects.

Methods: This FIH study was designed to determine the maximal tolerated dose (MTD) and recommended phase 2 dose (RP2D) to assess the tolerance, pharmacokinetics (PK) and pharmacodynamics profiles of YS110 and preliminary efficacy. YS110 were initially administered intravenously once every 2 weeks (Q2W) for three doses and then, based on PK data, once every week (Q1W) for five doses in patients with CD26-expressing solid tumours.

Results: Thirty-three patients (22 mesothelioma) received a median of 3 (range 1–30) YS110 infusions across six dose levels (0.1–6 mg kg^{-1}). MTD was not reached and two dose-limiting toxicities (infusion hypersensitivity reactions) led to the institution of a systemic premedication. Low-grade asthenia (30.3%), hypersensitivity (27.3%), nausea (15.2%), flushing (15.2%), chills (12.1%) and pyrexia (12.1%) were reported as ADRs. Pharmacokinetic parameters (AUC and C_{max}) increased in proportion with the dose. sCD26/DPPIV assays indicated CD26 modulation. Prolonged stable diseases were observed in 13 out of 26 evaluable patients.

Conclusions: YS110 is well tolerated up to 6 mg kg^{-1} Q1W, which has been defined as the RP2D, with encouraging prolonged disease stabilisations observed in a number of patients with advanced/refractory mesothelioma.

CD26 is a 110-kDa, type II transmembrane glycoprotein with known dipeptidyl peptidase IV (DPPIV, EC 3.4.14.5) activity in its extracellular domain and is capable of cleaving N-terminal dipeptides with either L-proline or L-alanine at the penultimate position (Torimoto *et al*, 1992; Ohnuma *et al*, 2008). CD26 activity is dependent on cell type and the microenvironment factors that

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can influence its multiple biological roles (Havre *et al,* 2008; Ohnuma *et al,* 2011). Robust evidence from our accumulating data indicates that CD26 has an important role in T-cell biology and overall immune function (Morimoto *et al,* 1989; Tanaka *et al*, 1993; Morimoto and Schlossman, 1998; Dang *et al,* 1990a, b; Hegen *et al,* 1997; Ohnuma *et al,* 2008).

CD26 is also expressed on various tumours such as malignant pleural mesothelioma (MPM), renal cell carcinoma (RCC), colorectal cancer (CRC), hepatocellular carcinoma, lung cancer, prostate cancer, gastrointestinal stromal tumour, thyroid cancer and haematologic malignancies such as T-anaplastic large cell lymphoma, T-lymphoblastic lymphoma and T-acute lymphoblastic leukaemia (Havre *et al,* 2008).

Our previous work demonstrated that CD26 is preferentially expressed in MPM cells but not in normal mesothelial cells (Amatya *et al,* 2011), and suggested that membranous expression of CD26 is of potential importance in the treatment of MPM patients (Aoe *et al,* 2012). More recently, we demonstrated that the CD26-positive population of $CD24^{+}CD9^{+}$ MPM cells exhibits the cancer stem cell features (Ghani *et al,* 2011; Yamazaki *et al,* 2012). We also reported robust *in vivo* data on the anti-tumour activity of anti-CD26 mAb in mouse xenograft models (Ho *et al*, 2001; Inamoto *et al,* 2006, 2007; Okamoto *et al,* 2014; Yamamoto *et al,* 2014).

YS110 is a recombinant DNA-derived humanised monoclonal antibody that selectively binds with high affinity to the extracellular domain of CD26.

The antibody is an IgG1 kappa with a molecular weight of 144 kDa and was humanised via an *in silico* design based on the amino-acid sequence of anti-human CD26 murine mAb (14D10), which inhibited tumour cell growth, migration and invasion, and enhanced survival of mouse xenograft models (Inamoto *et al,* 2006). The gene of YS110 is deposited to ATCC in designated with accession No. PTA-7695. The gene is preserved in DH5α *Escherichia coli* with plasmid having insert of heavy and light chain of a humanised monoclonal antibody against human CD 26 cDNA. The strain designation is S604069. YST-pABMC 148 ($\times$ 411). YS110 is produced by fermentation in mammalian cell (Chinese hamster ovary) suspension culture with the Glutamine Synthetase Expression System. *In vitro* pharmacological evaluation of YS110 demonstrated its selective binding to human CD26 on a number of human cancer cell lines and tissues, and no evidence for immune activation and no effect on DPPIV activity, while exhibiting direct cytotoxic effect on certain human CD26-positive cancer cell lines (Inamoto *et al,* 2006). In addition to antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity (Inamoto *et al,* 2006), YS110 induces tumour cell lysis *in vitro* via alternative original mechanisms: (i) a direct anti-tumour effect through the induction of cell cycle arrest by induction of $p27^{kip1}$ expression (Inamoto *et al,* 2006, 2007), (ii) following internalisation of the CD26-YS110 complexes, an inhibition of invasion and migration of tumours cells by decreased binding to the collagen/fibronectin microenvironment matrix (Inamoto *et al,* 2006, 2007) and (iii) the nuclear translocation of CD26 molecules by internalisation of the CD26-YS110 complexes to inhibit proliferation of MPM cells via suppression of *POLR2A* gene expression (Yamada *et al,* 2013). In further studies using preclinical models, *in vivo* administration of YS110 resulted in inhibition of tumour cell growth, migration and invasion, and enhanced survival of mouse xenograft models inoculated with RCC or MPM (Inamoto, *et al,* 2007; Okamoto *et al,* 2014; Yamamoto *et al,* 2014).

In addition to our robust *in vitro* and *in vivo* data on antibody-mediated dose-dependent tumour growth inhibition, YS110 exhibited excellent safety and pharmacological profiles in non-human primate models using single and repeated increasing intravenous doses. Considering the lack of T-cell proliferation and cytokine production *in vitro*, YS110 was therefore considered not to have an agonistic nor activating effect on human CD26-positive lymphocytes.

This first-in-human phase 1 clinical trial aims to evaluate the safety, pharmacokinetic/pharmacodynamic profiles and preliminary anti-tumour effects of YS110 in patients with CD26-expressing solid tumours and, particularly, refractory malignant mesothelioma, a tumour type in which successful therapeutic advances are expected to be warranted for a long time.

MATERIALS AND METHODS

Patients. Eligible patients were 18–80 years old with locally advanced, inoperable or refractory solid tumours that were histologically documented to express the CD26 molecule. Cancer histologies included mesothelioma (pleural or peritoneal) or other solid tumours such as non-small-cell lung carcinoma, RCC or hepatocellular carcinoma. All patients were in relapse following or were refractory to prior standard therapies (regardless of the number of prior treatment lines), with a progressive evaluable/measurable disease. Other key inclusion criteria included Eastern Cooperative Oncology Group (ECOG) performance status score $\leqslant 2$, adequate bone marrow, liver and renal function; at least 4 weeks from prior surgery, chemotherapy, external radiotherapy or immunotherapy (at least 6 weeks from prior nitrosoureas). All patients provided written informed consent. This study was conducted according to the Declaration of Helsinki and was approved by an ethics committee and the French National Drug Agency.

CD26 immunohistochemistry screening. Analysis of CD26 tumour expression for pre-screening was performed centrally at Gustave Roussy by conventional immunohistochemistry performed on formalin-fixed paraffin-embedded (FFPE) archival tumour samples using a anti-human CD26 goat polyclonal antibody (AF1180, R&D Systems, Minneapolis, MN, USA) as previously described (Aoe *et al,* 2012). Briefly, all patients were pre-screened for confirmed CD26-positive expression, defined as $\geqslant$20% of the tumour cells (1+, 2+ or 3+ intensity) and verified independently by two pathologists (P Vielh and T Yamada) (Figure 1).

Study design. This first-in-human study was designed as a classical 3 + 3 dose-escalating phase 1 trial of the single agent YS110 (Supplementary Table 1). The primary objective was to determine the maximum tolerated dose (MTD) based on the occurrence of dose-limiting toxicity (DLT) and a recommended phase 2 dose (RP2D). Initially, cohorts 1–4 each planned to enrol 3, and up to 6, patients sequentially to evaluate escalating YS110 doses at 0.1, 0.4, 1 and 2 mg kg^{-1} for a total of three infusions of YS110 on days 1, 15 and 29 (one treatment cycle, Q2W). On the basis of preliminary pharmacokinetics (PK) data, the protocol was then subsequently amended to allow patients to receive a total of five infusions of YS110 on days 1, 8, 15, 22 and 29 (one treatment cycle, Q1W) at 2, 4, and 6 mg kg^{-1}. Each cohort included a 24-h monitoring period following each infusion during the first cycle for evaluation of safety, DLTs and PK. Patients who completed one cycle and demonstrated a clinical response or stable disease could receive the second cycle of YS110 treatment cycles at the same dose and dosing frequency, until disease progression or a significant observed serious adverse event (SAE). If the patient continued the treatment, the second cycle was to begin initially 4 weeks (later amended to 2 weeks) after the last infusion of the first cycle (initially Day 57; after amendment, Day 43). For the subsequent cycles, the latency period between the last administration of a cycle and the beginning of the next cycle was 2 weeks. Secondary objectives of the study were to assess the safety and tolerability

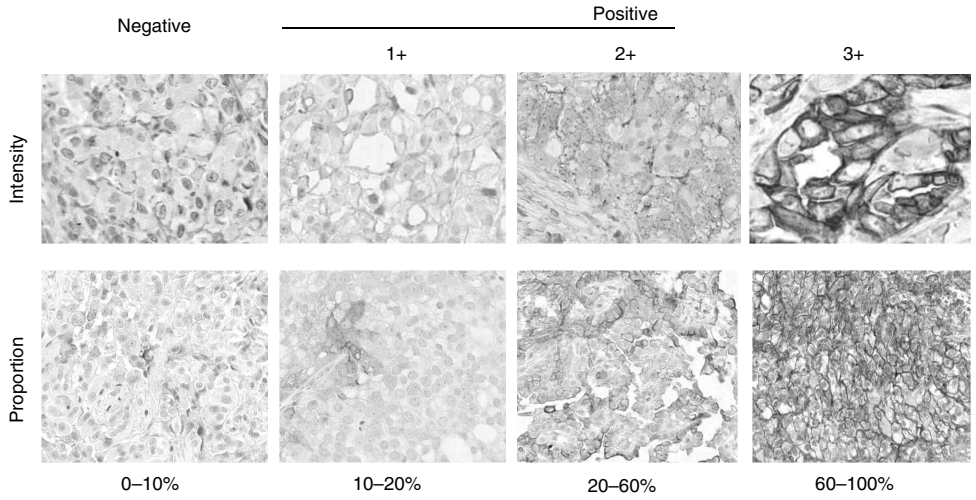

Figure 1. Evaluation of CD26 staining by immunohistochemistry in cases of mesothelioma.

profile, PK, pharmacodynamics, preliminary anti-tumour activity and to collect survival data.

Safety. All adverse events and SAEs occurring from the informed consent signature up to 30 days after the last dose were reported according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE v3.0). An adverse drug reaction (ADR) was defined as an adverse event (AE) documented as possibly, probably or definitely related to the study drug or with unknown relationship to the study drug. A treatment-emergent adverse event was defined as an AE with onset on or after the first infusion. The DLT period was 2 weeks after the first YS110 infusion, whatever the dose schedule. Dose-limiting toxicities were defined as any grade $\geqslant 3$ non-haematological toxicity or a haematological toxicity of grade $\geqslant 4$. This definition was later amended to exclude reversible grade 3 infusion reactions defined as allergic reaction/hypersensitivity, fever, pain, bronchospasm, wheezing or hypoxia, occurring during the first dose infusion and resolving with a reduced infusion rate, a stop of the infusion, supportive care and/or the administration of corticosteroids (Supplementary Table S2). In case of treatment discontinuation due to reversible grade 3 infusion reactions, the patient was to be replaced with a new patient of the same cohort.

Efficacy. Preliminary clinical efficacy was evaluated by radiological and tumour marker assessments performed at screening at Day 43 of each treatment cycle, and at end of treatment until progressive disease (PD) or withdrawal of consent. Tumour response was evaluated based on RECIST 1.0 criteria and defined as partial response (PR) or PD or stable disease (SD). Partial response was defined as $\geqslant 30\%$ decrease in the sum of the longest diameter (LD) of target lesions, PD was defined as $\geqslant 20\%$ increase in the sum of the LD of target lesions and stable disease was defined as neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD. Patients with mesothelioma were assessed according to modified RECIST criteria.

Pharmacokinetics. Serial blood samples were obtained at pre- and post-YS110 administration at Day 1, Day 8, Day 15 and Day 29 at specified time points throughout the study. Serum levels of YS110 were analysed by ELISA by Alta Intertek (San Diego, CA, USA). The method of blood sample collection is described in Supplementary Information.

Pharmacodynamics. All treated patients with appropriate post baseline samples were evaluable for pharmacodynamics analyses on blood samples collected at days 0 (baseline), 1 (post infusion), 2, 15 (pre- and post infusion), 29 (pre- and post infusion) for immunomonitoring and soluble CD26 (sCD26)/DPPIV (sDPPIV) activity assessments.

Immunomonitoring. Immunomonitoring was performed centrally in the translational research laboratory at Institut Gustave Roussy, France (F Farace).

Immunophenotyping was performed for the monitoring of peripheral blood lymphocyte (PBL) $CD26^+$ T (i.e., $CD3^+CD4^+$, $CD3^+CD8^+$) and NK (i.e., $CD3^-CD16^{+/-}CD56^+$) subpopulations by flow cytometry using fluorochrome-conjugated commercially available specific mAbs with relevant isotypic controls (all provided from Pharmingen, San Diego, CA, USA). To ensure the specificity of the CD26 staining in blood samples collected under YS110 treatment, competition and cross-blocking experiments using the two different CD26 mAb clones 5K78 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and M-A261 (BD Pharmingen, San Jose, CA, USA) were also performed.

Dosages of serum cytokines (i.e., interleukin 6 (IL-6), tumour necrosis factor α (TNF-α) and IL-2) were investigated by standard commercially available ELISA assays (R&D Systems).

Soluble CD26 (sCD26) and DPPIV activity assessments. Assays for soluble CD26 and DPP4 were developed in the Morimoto's Laboratory (Juntendo University, Japan) using anti-human CD26 murine mAbs that exhibit no cross-reactivity with the therapeutic humanised anti-CD26 mAb YS110 as described previously (Dong *et al*, 1998; Ohnuma *et al*, 2015a). The sampling times for sCD26 were identical to that for immunomonitoring.

Statistical analyses. Descriptive statistics were used to summarise the data. The patient sampling size was a function of the $3+3$ dose escalation schedule. The realised sample size was dependent on the number and pattern of observed DLTs. The maximum sample size was a consequence of the design's sampling requirements and the number of dose levels. In this trial with six dose levels, the maximum number of patients being exposed in each dose level was determined according to the DTL occurrence rate and confirmation of safety.

RESULTS

Screening for CD26 expression. A total of 136 patients signed an informed consent form and were screened for CD26 expression on a FFPE archival tumour tissue sample. Mesothelioma, which was previously reported as one of the CD26-expressing tumour types

was a main target indication with 60 patients screened ($n=60$, 53% were $CD26^+$ cases). Examples of CD26 immunohistochemistry and scoring are presented in Figure 1. Other tumour types screened included RCC ($n=18$, 71% $CD26^+$ cases), hepatocellular carcinoma ($n=7$, 22% $CD26^+$ cases) and various other tumour types ($n=31$, 13% $CD26^+$ cases). Overall, 56 (41%) patients were considered as $CD26^+$ with the mean percentage of CD26 expression on tumour cells of 69% (range 20–100%) in the tested samples.

Patient characteristics. Thirty-four $CD26^+$ patients (19 males and 15 females) were enroled in the study and treated at 5 investigational sites in France, with 33 patients being evaluable (19 males and 14 females). The most common tumour types were mesothelioma ($n=23$, 69.7%), RCC ($n=9$, 27.3%) and one patient had an urothelial carcinoma ($n=1$; 3.0%). All mesothelioma patients were diagnosed with the epithelioid histology and were all in advanced inoperable disease or refractory to previous line of treatment. Other tumour types were in stage III/IV Twenty-two patients were treated according to the Q2W schedule at 0.1 ($n=3$), 0.4 ($n=3$), 1 ($n=6$) and 2 mg kg^{-1} ($n=10$) and 11 according to the Q1W schedule at 2 ($n=4$), 4 ($n=3$) and 6 ($n=4$) mg kg^{-1} (Table 1). The median percentage of $CD26^+$ tumour cells in archival samples from the patients treated was 63% (range 25–100%).

Safety. Eighteen patients received at least one cycle of YS110 with three infusions per cycle and seven patients completed at least one cycle with five infusions per cycle. Nine patients did not complete the first cycle.

During the DLT observation period (i.e., 15 days following the first infusion), two DLTs were reported as serious unexpected adverse events (SUSARs) consisting of grade 3 infusion reactions (one patient at the Q2W 1 mg kg^{-1} dose level and another patient at the Q2W 2 mg kg^{-1} dose level) that completely resolved with supportive treatment, but led to permanent discontinuation of treatment. As both patients had a history of allergies, consequently, the protocol was subsequently amended to add clinically relevant allergies as a new excluding criterion, as well as the administration of a systematic steroid prophylaxis prior to each infusion to better control potential infusion reactions (chills, fever, flushing, hypotension and respiratory disorders). With these modifications, dose escalation was possible up to 6 mg kg^{-1} in four patients without DLTs. Per protocol, dose escalation was stopped at the highest level of 6 mg kg^{-1} without the MTD being achieved.

All patients had one or more AEs and 113 ADRs considered to be possibly related to YS110 were reported by 30 (90.9%) patients. The most frequently reported AEs regardless of potential relationship to YS110 were asthenia (54.5%) and aggravation of prior conditions (30.3%). Adverse effects (related or not to treatment) occurring in more than 10% of the patients are reported in Table 2 according to system organ class and preferred term. The majority of AEs were of mild (grade 1) or moderate (grade 2) severity. The most commonly reported grade $\geqslant 3$ AEs were dyspnoea (21.2%), hypersensitivity (15.2%), aggravation of prior conditions (15.2%), general physical health deterioration (12.1%) and hyperglycaemia (12.1%). Eight patients (24.2%) discontinued YS110 due to adverse events; most adverse events leading to discontinuation were considered unrelated to YS110, except for the two patients with infusion reactions considered as DLTs. Twenty-seven SAEs were reported in this study in 18 patients. Except for the two DLTs, all other SAEs, most commonly general physical health deterioration, were considered to be unrelated to YS110 but rather related to consequences of disease progression as assessed by the investigator, including the six patient deaths during the study.

No dose-dependent AEs were observed. Low-grade asthenia (30.3%), hypersensitivity (27.3%), nausea (15.2%), flushing (15.2%), chills (12.1%) and pyrexia (12.1%) were reported as ADRs.

No clinically significant abnormalities were observed in haematology and clinical chemistry laboratory parameters, as well as in ECG findings.

The main limiting toxicities in the study were infusion reactions, two being considered as DLTs leading to permanent discontinuation of treatment. Six severe hypersensitivity reactions were reported in five patients receiving a dose of 2.0 mg kg^{-1} and a severe anaphylactic reaction was reported in one patient receiving a dose of 1.0 mg kg^{-1}. These ADRs, occurring mainly at the first infusion, were reversible and manageable with curative corticosteroids and antihistaminic drugs, and further prevented by a systemic corticosteroids premedication. Overall, these ADRs that did not appear to be related either to the dose level of YS110 or to the Q2W or Q1W schedule.

Pharmacokinetics. Following single and repeat intravenous infusions of YS110, there was a trend toward decreasing clearance (CL), increasing half-life time ($T_{1/2}$) and increasing exposure (C_{max}, AUC_{0-168}, $AUC_{0-\infty}$) with increasing doses, suggesting that CL was saturating across the dose range studied. Mean volume of distribution (V_z, V_{ss}) was similar or slightly higher than human serum volume, indicative of YS110 being primarily found in serum and consistent with prior observations for monoclonal antibodies. In general, exposure increased as the dose increased. Although increases in C_{max} appeared to be dose proportional, increases in AUC_{0-168} and $AUC_{0-\infty}$ were greater than dose proportional, and this was consistent with the trend of decreasing CL with increasing doses (Table 3). In addition, YS110 PK parameters changed with repeat dosing. For doses ranging from 1 to 6 mg kg^{-1}, mean CL was ~1.1–1.6-fold higher on Day 1 (*vs* Day 29), whereas mean $T_{1/2}$ increased ~1.2–2.3-fold and exposure (AUC_{0-168}) increased ~1.3–1.8-fold on Day 29 *vs* Day 1 (Table 3).

Table 1. Baseline demographics and disease characteristics

Number of patients ($n=33$)		Schedule and dose levels (mg kg^{-1})						
Age, median (min–max) (yrs)	63 (41–76)							
ECOG PS 0/1/2 (%)	29/58/13							
Primary tumour type, *n* (%)		Q2W				Q1W		
		0.1	0.4	1	2	2	4	6
Mesothelioma (Meso)	22 (67)	3	2	2	6	3	2	4
Renal cell carcinoma (RCC)	10 (30)	—	1	4	4	—	1	—
Urothelial carcinoma (UTC)	1 (3)	—	—	—	—	1	—	—
Median number of prior therapies (min–max)	3 (1–11)							

Abbreviation: ECOG = Eastern Cooperative Oncology Group.

Table 2. Adverse events (all grades) reported by ⩾10% of patients overall in any cohort

	Dose levels (mg kg^{-1})							
Preferred term (CTCAE v3) number of patients (%)	**0.1 (*n* = 3)**	**0.4 (*n* = 3)**	**1.0 (*n* = 6)**	**2.0 (*n* = 14)**	**4.0 (*n* = 3)**	**6.0 (*n* = 4)**	**All AEs/grade 3–4 (*n* = 33)**	**All ADRs/grade3–4 (*n* = 33)**
Asthenia	3 100.0)	2 (66.7)	0 (0.0)	8 (57.1)	2 (66.7)	3 (75.0)	18 (54.5)	10 (30.3)
Condition aggravated	1 (33.3)	0 (0.0)	1 (16.7)	4 (28.6)	1 (33.3)	3 (75.0)	10 (30.3)/5 (15.2)	
Pyrexia	2 (66.7)	1 (33.3)	1 (16.7)	3 (21.4)	0 (0.0)	1 (25.0)	8 (24.2)	4 (12.1)
Chest pain	1 (33.3)	1 (33.3)	1 (16.7)	2 (14.3)	0 (0.0)	0 (0.0)	5 (15.2)	
Chills	2 (66.7)	1 (33.3)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	4 (12.1)	4 (12.1)
General health deterioration	0 (0.0)	0 (0.0)	0 (0.0)	2 (14.3)	1 (33.3)	1 (25.0)	4 (12.1)/4 (12.1)	
Constipation	2 (66.7)	0 (0.0)	1 (16.7)	5 (35.7)	0 (0.0)	1 (25.0)	9 (27.3)	
Nausea	0 (0.0)	1 (33.3)	1 (16.7)	3 (21.4)	2 (66.7)	1 (25.0)	8 (24.2)	5 (15.2)
Vomiting	1 (33.3)	1 (33.3)	2 (33.3)	2 (14.3)	1 (33.3)	0 (0.0)	7 (21.2)	
Diarrhoea	0 (0.0)	2 (66.7)	1 (16.7)	1 (7.1)	0 (0.0)	1 (25.0)	5 (15.2)	
Dyspnoea	2 (66.7)	1 (33.3)	3 (50.0)	5 (35.7)	0 (0.0)	3 (75.0)	14 (42.4)/7 (21.2)	
Hyperglycaemia	0 (0.0)	0 (0.0)	1 (16.7)	3 (21.4)	2 (66.7)	2 (50.0)	8 (24.2)/4 (12.1)	
Decreased appetite	0 (0.0)	1 (33.3)	1 (16.7)	3 (21.4)	1 (33.3)	1 (25.0)	7 (21.2)	
Headache	1 (33.3)	2 (66.7)	0 (0.0)	3 (21.4)	1 (33.3)	0 (0.0)	7 (21.2)	
Flushing	0 (0.0)	0 (0.0)	0 (0.0)	1 (7.1)	2 (66.7)	3 (75.0)	6 (18.2)	5 (15.2)
Hypersensitivity	0 (0.0)	0 (0.0)	1 (16.7)	8 (57.1)	0 (0.0)	0 (0.0)	9 (27.3)/5 (15.2)	9 (27.3)/5 (15.2)
Weight decreased	0 (0.0)	1 (33.3)	0 (0.0)	5 (35.7)	0 (0.0)	0 (0.0)	6 (18.2)	

Abbreviations: ADR = adverse drug reactions; AE = adverse events; *N* = number of patients. A subject with more than one finding in a specific category was only counted once; percentages are based on the total number of subjects in each treatment group. The table is sorted by descending subject count. Infusion reactions related to YS110 treatment were further prevented by corticosteroids premedication.

Table 3. Cycle 1 pharmacokinetics parameters (mean ± s.d.) for YS110 administration

Dose (mg kg^{-1})	Dose Schedule	Day	$T_{1/2}$ (h)	C_{max} (μg ml^{-1})	AUC_{0-168} (h × μg ml^{-1})	$AUC_{0-\infty}$ (h × μg ml^{-1})	CL (ml h^{-1} kg^{-1})
0.4	Q2W	1	ND	ND	ND	ND	ND
		29	14.8 ± ND	5.85 ± ND	143 ± ND	145 ± ND	2.79 ± ND
1	Q2W	1	26.4 ± ND	22.7 ± 5.68	768 ± 73.3	692 ± ND	1.44 ± ND
		29	ND	43.5 ± 29.3	979 ± NR	ND	1.05 ± NR
2	Q2W	1	36.4 ± 12.2	39.0 ± 9.94	1710 ± 360	1810 ± 472	1.16 ± 0.245
		29	43.1 ± 12.6	40.1 ± 10.4	2080 ± 943	2280 ± 1110	1.03 ± 0.435
2	Q1W	1	24.5 ± 4.59	30.8 ± 4.20	1180 ± 243	1200 ± 251	1.72 ± 0.334
		15	31.5 ± NR	67.3 ± NR	2150 ± NR	2230 ± NR	1.22 ± NR
		29	29.8 ± NR	27.5 ± 20.1	1650 ± NR	1720 ± NR	1.33 ± NR
4	Q1W	1	46.6 ± 1.69	72.5 ± 27.1	4340 ± 1030	4740 ± 1140	0.876 ± 0.206
		15	70.4 ± NR	82.4 ± NR	6000 ± NR	7450 ± NR	0.678 ± NR
		29	76.2 ± ND	98.4 ± ND	7320 ± ND	9340 ± ND	0.547 ± ND
6	Q1W	1	67.8 ± 13.8	150 ± 22.9	10 300 ± 1800	12 800 ± 3250	0.490 ± 0.116
		15	93.7 ± 27.4	182 ± 17.2	15 700 ± 3470	22 800 ± 8250	0.393 ± 0.0793
		29	154 ± NR	205 ± 23.6	18 400 ± 4320	39 700 ± NR	0.340 ± 0.0814

Abbreviations: AUC = area under the curve; CL = clearance; ND = not determined; NR = not reported.

For the initial dose levels (0.1, 0.4, 1 and 2 mg kg^{-1}), YS110 was administered on a Q2W schedule on days 1, 15 and 29. As expected, the maximum concentrations of YS110 (C_{max}) on days 1 and 29 were proportional to the dose levels. However, calculated half-lives (1–2 days) were shorter than expected for a humanised antibody and serum concentrations were at or below detectable levels (0.4 μg ml^{-1}) by 1 week post infusion. To maintain measurable YS110 trough concentrations between doses, dosing was increased to five doses on a Q1W schedule on days 1, 8, 15, 22 and 29 for dose levels 2, 4 and 6 mg kg^{-1}. C_{max} values on day 1 were roughly proportional to dose level. Mean half-lives increased with increasing dose levels, rising significantly from day 1 to day 15 to day 29 in the 2, 4 and 6 mg kg^{-1} cohorts ($\sim$1, $\sim$2 and $\sim$3 days, respectively), and resulting in drug accumulation between the doses (Table 3).

Impact from exposure to YS110 on anti-drug antibody (ADA) response appeared to be most pronounced in the 0.4 mg kg^{-1} dose cohort. The two subjects who were ADA positive on Day 29 of Cycle 1 had notably reduced exposure relative to the one ADA negative subject. There were no ADAs detected in the higher dose groups.

Efficacy. A secondary objective of the study was to evaluate for the potential anti-tumour activity of YS110 according to RECIST 1.0 criteria (or modified RECIST criteria for mesothelioma). No objective response was observed in the treated patients. However, stable disease per RECIST criteria as the best response was observed in 13 out of the 26 evaluable patients following the first cycle of treatment, as shown in the accompanying Waterfall Plot Chart (Supplementary Figure S2). Overall median PFS was 43 days

Table 4. Time on YS110 treatment and median PFS of treated patients

Cohorts	Dose levels (mg kg^{-1})	Cancer types (*n*)	Median infusions *N* (min–max)	Median PFS days (min–max)
Q2W	0.1	Meso (3)	3 (3)	42 (41–42)
Q2W	0.4	Meso (2) RCC (1)	18 (3–20)	223 (40–273)
Q2W	1.0	Meso (2) RCC (4)	3 (1–3)	40 (28–59)
Q2W	2.0	Meso (6) RCC (4)	3 (1–27)	57 (13–399)
Q1W	2.0	Meso (3) UTC (1)	5 (1–20)	47 (5–184)
Q1W	4.0	Meso (3)	4 (3–5)	32 (22–59)
Q1W	6.0	Meso (4)	17.5 (1–30)	58 (15–258)
All	All	All	4 (1–30)	43 (5–399)

Abbreviations: Meso = malignant mesothelioma; PFS = progression-free survival duration; RCC = renal cell carcinoma; UTC = urothelial carcinoma.

(Table 4) as determined by a Kaplan–Meier Plot (Supplementary Figure S3). However, PFS of 184–399 days was observed in 7 patients (five cases of mesothelioma and two cases of RCC) out of 26 cases (mesothelioma: 19 cases, RCC: 6 cases and urinary tract carcinoma: 1 case). Table 4 summarises the median number of infusions administered and median PFS according to the different dose levels and schedule.

Pharmacodynamics

Immunomonitoring. The CD26 immunophenotyping on peripheral T and NK lymphocyte subpopulations under YS110 treatment was validated by testing two different anti-CD26 mAb clones. Indeed, in the first 12 patients tested using the M-A261 mAb (BD Pharmingen), we observed a dramatic decrease of CD26$^+$ cells consequently to YS110 administration, suggesting the lack of detection of the CD26 epitope due to YS110 binding. These results (not shown), led us to test in parallel another clone 5K78 (Santa Cruz Biotechnology) showing that CD26$^+$ cells were still detectable under YS110 treatment. The validation of this anti-CD26 mAb was ensured by competition and cross-blocking experiments using increasing dilutions of YS110 in the CD26 immunophenotyping assay demonstrating no modulation of CD26$^+$ subpopulations using the 5K78 clone in contrast to the first clone tested.

At baseline in our series of patients, the mean ± s.d. ($n = 33$) absolute values ($\times 10^6$ per ml) of lymphocytes populations were 0.94 (65.7%) ± 0.64 for CD3$^+$CD4$^+$, 0.33 (34.5%) ± 0.26 for CD3$^+$ CD8$^+$ T cells and 0.17 (8.2%) ± 0.17 for CD3$^-$CD16$^{+/-}$CD56$^+$ NK cells. The mean percentage of CD26$^+$ subpopulations in the T-CD4, T-CD8 and NK cells was 24.7%, 8.2% and 5.2%, respectively with a significant inter-patient variability. After YS110 infusions, the levels of the various PBL subpopulations decreased at day 1 and 2 (i.e., 24–48 h following YS110 infusions), with a subsequent recovery at day 15 and 29 pre-infusion samples in most patients (Supplementary Table S3). This decline in different PBL subpopulations was more frequently observed in patients who received higher doses (2, 4 and 6 mg kg^{-1}) of YS110. However, this trend was not statistically significant considering the inter-individual variations across the different dose levels.

Serum cytokines. At baseline and during YS110, no serum IL-2 production was detectable in any of the patients. Significant rise of the pro-inflammatory cytokines IL-6 and TNF-α was detected at day 1 and 2 following the first infusion of YS110 at 0.4, 1 and 2 mg kg^{-1} at various levels, including the first patient with a grade 3 infusion reactions DLT (at the Q1W 1 mg kg^{-1} dose level). Different kinetics of serum IL-6 and TNF-α production were observed in patients receiving 2 mg kg^{-1} of YS110, whereas low or undetectable levels were observed in patients receiving 4.0 and 6.0 mg kg^{-1} of YS110, likely due to the administration of systemic steroid prophylaxis.

sCD26 and DPPIV activity in sera. To determine serum levels of sCD26 protein and DPPIV enzyme activity in each cohort before and after administration of YS110, we developed an in-house ELISA assay for sCD26 and DPPIV using anti-human CD26 murine mAbs exhibiting no cross-reactivity with the therapeutic humanised anti-CD26 mAb YS110 (Dong *et al,* 1998; Ohnuma *et al,* 2015a).

As shown in Figure 2A, an increase in YS110 infusion dose was associated with decreased serum sCD26 level, particularly in the cohorts of patients treated at 2, 4 and 6 mg kg^{-1}, with ~80% decrease in sCD26 level. Moreover, as CD26 level reflects DPPIV enzyme activity in the serum, a similar reduction in DPPIV enzyme activity was observed (Figure 2B).

DISCUSSION

YS110 is the first, and currently the only CD26-directed mAb in clinical trial. This FIH study demonstrates that YS110 therapy exhibits a favourable safety profile and results in encouraging disease stabilisation in heavily pretreated CD26-positive MPM or RCC patients who had previously progressed on conventional standard therapies.

The spectrum of AEs, the most common of which were low-grade asthenia, hypersensitivity, chills, pyrexia, nausea, vomiting and headache, was similar to that previously described with humanised mAbs treatment (Scott *et al,* 2012). Two DLTs were reported as SUSARs consisting in grade 3 infusion reactions that resolved with supportive treatment. As both patients had a history of allergies, the protocol was subsequently amended to include clinically relevant allergies as a new excluding criterion, and the administration of systemic steroid prophylaxis has been implemented prior to each infusion at all cycles to better control such infusion reactions. In this study, high rate of hyperglycaemia was observed in patients in the top dose cohorts, consistent with the well-known effect of corticosteroid on glucose levels. However, hyperglycaemia resolved soon after cessation of steroid administration, whereas YS110 was detected in sera, with decreased serum DPPIV activity. Taken together, it is probable that premedicated corticosteroid, but not YS110, induces the hyperglycaemia observed in the patient cohorts receiving higher drug doses. With these new modifications, dose escalation up to 6 mg kg^{-1} was possible as in four patients in this cohort were treated without DLTs. Per protocol, 6 mg kg^{-1} was the highest dose level tested although the MTD was not achieved. The dose level of 6.0 mg kg^{-1} of YS110 was decided by study investigators to be the RP2D.

Total lymphocyte counts, as well as levels of CD26$^+$ lymphocytes, fell at Day 2 below the baseline levels. However, total lymphocyte counts recovered to reach baseline level at Day 8 and thereafter. These data indicate that YS110 administration resulted in a decrease in levels of peripheral lymphocytes including the CD26-positive lymphocyte subset soon after drug administration, and it is probable that YS110-mediated suppression of peripheral lymphocyte levels, including the CD26-positive subset, resolved by as early as Day 8. Moreover, we observed a decrease in the level of the CD26-positive subset of peripheral lymphocytes following administration of YS110 alone without steroid prophylaxis in the 0.1–2.0 mg kg^{-1} cohorts, as shown in Supplementary Table S3. Therefore, it is conceivable that YS110, but not premedication steroid, was responsible for the temporary effect on CD26-positive lymphocyte counts.

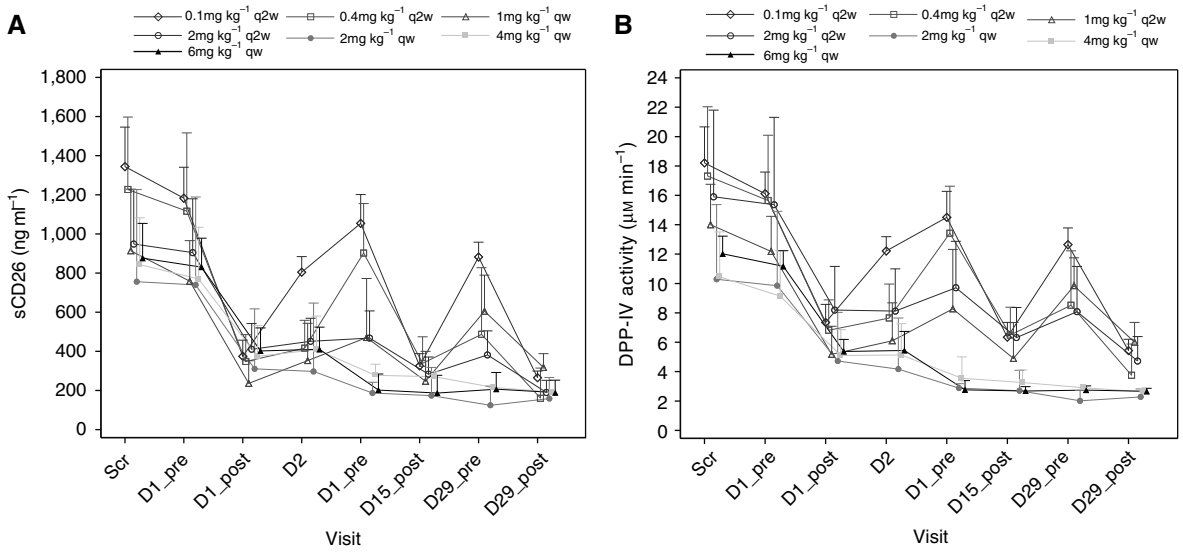

Figure 2. Changes of serum CD26/DPPIV levels following YS110 treatment: soluble CD26 (**A**) and DPPIV activity (**B**) mean ± s.d. at cycle 1 at the different dose levels.

CD26 is also present in serum and other body fluids in a truncated form as sCD26/DPPIV, and our data also indicated that DPPIV enzyme activity decreased with increasing doses of YS110 (Figure 2B). Although DPPIV inhibitors are clinically used as oral hypoglycaemia agents (Barreira da Silva *et al,* 2015), hypoglycaemia was not observed during YS110 administration. Of note is the fact that >80% inhibition of serum DPPIV activity was obtained 24 h after oral administration of clinically available DPPIV inhibitors (drug information published by each pharmaceutical company of sitagliptin, vildagliptin, saxagliptin and etc.), a level of inhibition comparable to that seen in patients treated with YS110. Our current data would therefore indicate that YS110 therapy is tolerable in the clinical setting.

The only treatment with level-one level of evidence for improving clinical outcome is the regimen consisting of a platinum doublet with an antifolate (van Meerbeeck *et al,* 2005). With this combined chemotherapy, patients with good performance status have a median overall survival of ~1 year, and a median PFS of <6 months. There is currently no standard second-line treatment with demonstrated ability to prolong survival. Importantly, patients who failed this first-line therapy have an extremely short survival (3.4 months), and are exposed to potentially life-threatening toxicities unnecessarily (Blayney *et al,* 2012). Due to the lack of efficacy of conventional therapeutic approaches, it is potentially significant that treatment with YS110 results in encouraging disease stabilisation with a median PFS of 32 weeks (26–57 weeks) in seven heavily pretreated CD26-positive patients who previously progressed on conventional therapies (Supplementary Figures S2 and S3).

Recent work has demonstrated the functional role of DPPIV-mediated posttranslational modification of chemokines in regulating tumour immunity through its interaction with its substrate CXCL10 (Ohnuma *et al,* 2015b). Preservation of the full length, bioactive CXCL10 by DPPIV inhibition using the DPPIV inhibitor sitagliptin resulted in increased level of $CXCR3^+$ effector T cells in the tumour microenvironment and subsequent tumour growth reduction (Ohnuma *et al,* 2015b). In view of these recent findings, data from our current trial showing that serum DPPIV activity was decreased following treatment with YS110 in a dose-dependent manner (Figure 2B). It would suggest that anti-tumour activity via DPPIV inhibition may constitute yet another mechanism of action for the anti-tumour activity of YS110, in addition to the mechanisms of action discussed above (Hatano *et al,* 2015).

We recently reported that CD3/CD26 costimulation induced the development of a human type 1 T_{reg} cells from $CD4^+$ T cells with high level of IL-10 production and lymphocyte activation gene 3 (LAG3) expression (Nguyen and Ohashi, 2015). Preclinical models showed that antibody-mediated blocking of LAG3 as potential anti-cancer therapy led to enhanced activation of antigen-specific T cells at the tumour sites and disruption of tumour growth (Nishikawa *et al,* 2015). Moreover, anti-LAG3/anti-PD-1 antibody treatment cured most mice of established tumours that were largely resistant to single antibody treatment (Nishikawa *et al,* 2015). Taken together, it is conceivable that CD26 itself may be a functional inhibitory molecule of an immune checkpoint system in certain disease conditions, similar to LAG3 or PD-1. In this scenario, CD26 blockade by YS110 may potentially function as an immune checkpoint blockade therapy, which can mediate anti-tumour activity in CD26-negative as well as CD26-positive cancers.

Microarray analyses of MPM cell lines treated with YS110 indicated that anti-CD26 mAb therapy resulted in the down-regulation of cytochrome *c* oxidase polypeptide I and II, and metallothione molecules that confer resistance to apoptosis or chemotherapeutic agents (Aoe *et al,* 2012). In addition, suppression of drug-resistant-related transporters, DNA repair enzyme and oncogenic protein expression was observed (Aoe *et al,* 2012). These preclinical results suggest that YS110 can function synergistically with other antineoplastic agents such as selected chemotherapeutic drugs to inhibit tumour growth.

Tumour surface expression of CD26/DPP4 may also have direct biological effects on the malignant behaviour of tumours. In several human malignancies including colorectal CRC, chronic myeloid leukaemia, gastric adenocarcinoma and MPM, CD26/DPP4 expression is reported to be a marker of cancer stem cells (Pang *et al,* 2010; Ghani *et al,* 2011; Yamazaki *et al,* 2012; Hatano *et al,* 2014; Herrmann *et al,* 2014; Davies *et al,* 2015). Given the potential role of CD26 surface expression in cancer biology, YS110 therapy may also influence tumour growth through its potential effect on the cancer stem cells of selected tumours.

In conclusion, our FIH study showed that YS110 therapy is generally well tolerated up to 6 mg kg^{-1} Q1W, which has been

defined as the RP2D and results in encouraging disease stabilisation in a number of patients with advanced/refractory CD26-expressing cancers. Our findings also suggest that further clinical development of YS110 such as its use as part of combination therapies with other antineoplastic agents is warranted.

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CONFLICT OF INTEREST

Yutaro Kaneko is an employer of Y's AC Co., Ltd, Japan, Thomas Podoll is an employer of Y's Therapeutics Inc., USA, and Yu Kuramochi and Itaru Miyashita are the employees of Kissei Pharmaceutical Co., Ltd, Tokyo, Japan. Chikao Morimoto, Kei Ohnuma and Nam H Dang are stock holders of Y's AC Co., Ltd. The remaining authors declare no conflict of interest.

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