Entity Hepatocellular carcinoma

Note In normal liver as well as cirrhotic and steatotic liver, CD26 is expressed in the bile canalicular domains of hepatocellular membranes. In hepatocellular carcinoma (HCC), CD26/DPPIV activity was lost (2/25 cases) or had altered distribution (23/25 cases). Gaetaniello and colleagues found Tyr phosphorylation of several proteins after antibody binding to CD26 in HCC cell lines. This effect was inhibited by CD45 Tyr phosphatase. They found that a protein or proteins with kinase activity were associated with CD26. CD26 protects against apoptosis in Jurkat T-cells, while in the human hepatoma PLC/PRF/5 cells CD26 antibody binding is pro-apoptotic. CD26 expression is also pro-apoptotic in HepG2 cells.

Entity Glioma

Note Low CD26/DPPIV activity is found in poorly differentiated gliomas and high activity is found in differentiated gliomas. Differentiated gliomas have higher levels of membrane-associated isoforms of CD26/DPPIV. Sedo and colleagues studied "DPPIV activity and or structure homologs" (DASH) in 5 glioma cell lines of varying grade. They concluded that there was "no simple correlation with the degree of malignancy of the original donor tumor or morphologic phenotype...". They did note that changes in DASH composition as detected by non-denaturing polyacrylamide gel electrophoresis may reflect changes in substrate specificity and regulate changes in glioma activity.

Entity Prostate cancer

Note CD26/DPPIV biochemical activity was twice as high in prostate cancer compared to benign prostate hyperplasia (BPH) tissues. CD26/DPPIV activity was also increased in BPH adjacent to cancerous prostate tissue. DPPIV activity was present in epithelial but not stromal BPH and cancer tissues. This suggests that paracrine factors produced by prostate cancer cells may modulate the local microenvironment to increase cancer growth. Johnson et al. found that lung endothelial cells with metastatic prostate cancer cells were enriched for CD26 compared to non-metastatic prostate cancer cells. However, Wesley et al. suggested that CD26 inhibits the "malignant phenotype" of prostate cancer cells by inhibiting the FGF signaling pathway. Wilson et al. examined DPPIV activities in different prostatic tissue zones and in prostatic expressed secretions in relation to the presence of cancer. They found that expressed prostate secretions in patients with cancer were higher than in men without cancer. DPPIV activities in the transitional and especially the peripheral zone biopsies were higher in cancer patients. They concluded that secreted DPPIV originates in the transitional and peripheral zones. Measuring DPPIV levels in prostate secretion or in post-digital rectal prostate examination urine may be a useful for tumor marker for prostate cancer. Gonzalez-Gronow et al. found that in addition to its ability to inhibit tumor vascularization, angiostatin 2ε may also directly block prostate cancer metastasis by binding to CD26 on the surface of the 1-LN prostate cancer cell line. CD26/DPPIV association with plasminogen may lead to signal transduction that regulates expression of MMP-9 in prostate cancer cells. CD26 inhibitors may inhibit prostate cancer by blocking a CD26/DPPIV mediated signal transduction pathway that regulates MMP-9 expression by prostate cancer cells.

Entity Renal cancer

Note CD26 expression has previously been noted in renal cell carcinoma, with unclear significance. CD26 has a high level of surface expression on the renal cell carcinoma cell lines Caki-1, Caki-2, VRMRC-RCW, and ACHN. Inamoto and colleagues showed that anti-CD26 monoclonal antibody 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). They also found that anti-CD26 monoclonal antibody therapy attenuated Akt activity and internalized cell surface CD26 leading to decreased CD26 binding to collagen and fibronectin. More importantly, they showed that anti-CD26 monoclonal antibodies inhibited human renal cell carcinoma cell growth in a mouse xenograft model, with prolongation of survival. These results suggest that CD26 is a good target for renal cell carcinoma.

Entity Thyroid cancer

Note Benign thyroid tissue is usually negative for CD26 expression, but CD26 expression has been observed in thyroid cancer. This difference has been exploited as a diagnostic marker for the differential diagnosis of benign thyroid disease versus thyroid cancer. Kholová et al. suggest that CD26 positivity is limited to well-differentiated thyroid carcinomas including papillary carcinoma and is of limited value for diagnosing follicular and oncocytic thyroid malignancies.

Entity Melanoma

Note CD26 is highly expressed in normal melanocytes, but not in melanoma cells, suggesting CD26 expression is lost in malignant transformation. Loss of CD26 was also associated with development of specific chromosome abnormalities. When CD26/DPPIV is expressed in melanoma cells using a tetracycline-inducible expression system, melanoma growth was suppressed in the clones of cell line expressing higher levels of CD26. CD26 expression also decreased the melanoma growth in soft agar - indicating a CD26 association with anchorage-independent growth. Also, CD26 can induce MMP-9 expression in cancer cells, which may facilitate metastasis. Pethiyagoda et al. confirmed that CD26/DPPIV inhibits invasion of malignant melanoma cell lines by transfecting CD26 into melanoma cell lines. Invasion in Matrigel was decreased by 75%. This was also true for CD26 mutant transfectants that either lacked the extracellular protease activity (Ser630Ala) or the six amino acid cytoplasmic domain - demonstrating that neither DPPIV activity or the six amino acid C-terminal domain were necessary to inhibit invasiveness. Expression of either wild-type CD26 (DPPIV positive) or mutant CD26 (DPPIV negative) rescued expression of the cell surface Ser protease FAP-α. FAP-α can form a heterodimer with CD26 and may play a role in regulating melanocyte/melanoma growth. CD26 can inactivate circulating growth hormone-releasing factor (GHRF), so decreased CD26 may result in cancer growth by increased GHRF. A recent study found no effect of IFN-α on DPPIV activity in 18 patients with high-risk melanoma.

Entity Ovarian cancer

Note Expression of CD26 varies in ovarian cancer cell lines. CD26 is negatively correlated with ovarian cancer invasive potential. Specifically, overexpression of CD26 in ovarian cancer leads to increased E-cadherin and tissue inhibitors of MMPs, resulting in decreased invasive potential. CD26 transfection in ovarian cancer cell lines decreased intraperitoneal dissemination and prolonged survival in vivo in mice.

Entity Malignant mesothelioma

Note Malignant pleural mesothelioma (MPM) is an aggressive cancer arising from the mesothelial cells lining the pleura. Morimoto et al. showed that CD26 is preferentially expressed on malignant mesothelioma cells but not on normal mesothelial cells, and suggested that membranous expression of CD26 indicates an importance in treatment of patients with MPM. More importantly, humanized anti-CD26 antibody inhibited growth of malignant mesothelioma cells and induced long term survival of tumor-

transplanted SCID mice. In addtion, it has been shown that cells from certain CD26-positive mesothelioma cell lines appeared to include the cancer stem cell characteristics for malignant mesothelioma in addition to CD24 and CD9-positive cells. Furthermore, Morimoto et al. showed that the CD26 molecule is expressed on the cell membrane of the epithelial and biphasic, but not the sarcomatoid, type of mesothelioma. Importantly, treatment outcome prediction study showed that CD26 membrane expression on MPM was closely correlated with disease responsiveness to chemotherapy. Meanwhile, in vitro studies showed that mesothelioma cells expressing high level of CD26 displayed high proliferative activity, and microarray analysis of CD26 knockdown and CD26-transfected mesothelioma cells showed that CD26 expression was closely linked to expression of genes contributing to cell proliferation, cell cycle regulation, drug-induced apoptotic action, and chemotherapy resistance. These data therefore strongly suggest that the CD26 molecule is a good therapeutic target for MPM and a clinically significant biomarker for the prediction of response to chemotherapy for MPM.

Entity Gastric gastrointestinal stromal tumors

Note In an immunohistochemical analysis of 152 patients with gastric gastrointestinal stromal tumors (GIST), CD26 expression was found to be associated with a poorer overall survival.

Entity Rheumatoid arthritis

Note Rheumatoid arthritis (RA) is a chronic, inflammatory autoimmune disease and is characterized by progressive invasion of synovial fibroblasts into the articular cartilage and erosion of the undelying bone, followed by joint destruction. In antigen-induced arthritis mouse model, $DPPIV^{-/-}$ mice showed more severe arthritis, and enzymatic activity levels of DPPIV in the plasma were significantly decreased in RA patients. Morever, other investigators reported that inhibition of DPPIV and FAP increases cartilage invasion by RA synovial fibroblasts. However, CD26/DPPIV regulates biologic processes that are unrelated to its peptidase activity, e.g., cellular adhesion, cell differentiation, and activation via downstream signaling cascade. $CD26^{+}$ T cells induce the inflammation and tissue destruction characteristic of RA by migrating to and being active in the rheumatoid synovium. Cordero et al. studied IL-12, IL-15, soluble CD26, and ADA serum levels from 35 patients with active and inactive RA as well as those of controls. 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. Mavropoulos et al. found that anti-TNF-α therapy increases DPPIV activity and decreases autoantibodies to the chaperone protein Bip (GRP78) and phosphoglucose isomerase in 15 patients with RA. DPPIV inhibitors inhibit a rat model of rheumatoid arthritis in a dose-dependent manner. Ohnuma et al. described $CD26^{+}$ T cells infiltrating the rheumatoid synovium using immunohistochemical studies. They found high expression of caveolin-1 in the rheumatoid synovium vasculature and synoviocytes. These data suggest that the CD26-caveolin-1 upregulation of CD86 on activated monocytes leads to antigen-specific T-cell activation in rheumatoid arthritis. DPPIV inhibitors may be useful for suppressing the immune system in rheumatoid arthritis and other autoimmune diseases.

Entity Inflammatory bowel diseases

Note Crohn's disease and ulcerative colitis are categorized as inflammatory bowel disease (IBD), being characterized by chronic remittent or progressive inflammatory conditions that may affect the entire gastrointestinal tract and the colonic mucosa, respectively, and are associated with an increased risk for colon cancer. Sera from IBD patients contain lower levels of circulating DPPIV activity, while membrane expression of CD26/DPPIV on T cells isolated from IBD patients is higher than healthy controls. These clinical observations indicate that CD26/DPPIV might play a significant role in perpetuating the inflammatory response associated with IBD.

Entity Systemic lupus erythematosus

Note Serum levels of soluble CD26 and its specific DPPIV activity were significantly decreased in patients with systemic lupus erythematosus (SLE), and were inversely correlated with SLE disease activity index score, but not with clinical variables or clinical subsets of SLE. More recently, Lam et al. reported that CD26 expression on invariant natural killer cells of SLE patients is decreased significantly than that of healthy controls.

External links

	Nomenclature
HGNC (Hugo)	DPP4 3009
Entrez_Gene (NCBI)	DPP4 1803 dipeptidyl-peptidase 4
	Cards
Atlas	**DPP4ID40360ch2q24**
GeneCards (Weizmann)	DPP4
Ensembl (Hinxton)	ENSG00000197635 [Gene_View] chr2:162848755-162931052 [Contig_View] DPP4 [Vega]
AceView (NCBI)	DPP4
Genatlas (Paris)	DPP4
SOURCE (Stanford)	NM_001935
	Genomic and cartography
GoldenPath (UCSC)	DPP4 - 2q24.2 chr2:162848755-162931052 - 2q23-qter [Description] (hg19-Feb_2009)
Ensembl	DPP4 - 2q23-qter [CytoView]
Mapping of homologs : NCBI	DPP4 [Mapview]
OMIM	102720
	Gene and transcription
Genbank (Entrez)	AB451339 AB451488 AK314798 AY429531 AY429532
RefSeq transcript (SRS)	NM_001935
RefSeq transcript (Entrez)	NM_001935
RefSeq genomic (SRS)	AC_000134 NC_000002 NC_018913 NT_005403 NW_001838860 NW_004078008

RefSeq genomic (Entrez)	AC_000134 NC_000002 NC_018913 NT_005403 NW_001838860 NW_004078008
Consensus coding sequences : CCDS (NCBI)	DPP4
Cluster EST : Unigene	Hs.368912[SRS] Hs.368912 [NCBI]
CGAP (NCI)	Hs.368912
Alternative Splicing : Fast-db (Paris)	GSHG0018103
Alternative Splicing Gallery	ENSG00000197635
Gene Expression	DPP4 [NCBI-GEO] DPP4 [EBI - ARRAY_EXPRESS]
	Protein : pattern, domain, 3D structure
UniProt/SwissProt	P27487 (SRS) P27487 (Uniprot)
NextProt	P27487
With graphics : InterPro	P27487
Splice isoforms : SwissVar	P27487(Swissvar)
Domaine pattern : Prosite (SRS)	PRO_ENDOPEP_SER (PS00708)
Domaine pattern : Prosite (Expaxy)	PRO_ENDOPEP_SER (PS00708)
Domains : Interpro (SRS)	Pept_S9_AS Peptidase_S9 Peptidase_S9B
Domains : Interpro (EBI)	Pept_S9_AS Peptidase_S9 Peptidase_S9B
Related proteins : CluSTr	P27487
Domain families : Pfam (SRS)	DPPIV_N (PF00930) Peptidase_S9 (PF00326)
Domain families : Pfam (Sanger)	DPPIV_N (PF00930) Peptidase_S9 (PF00326)
Domain families : Pfam (NCBI)	pfam00930 pfam00326
DMDM	1803
Blocks (Seattle)	P27487
PDB (SRS)	1J2E 1N1M 1NU6 1NU8 1PFQ 1R9M 1R9N 1RWQ 1TK3 1TKR 1U8E 1W11 1WCY 1X70 2AJL 2BGN 2BGR 2BUB 2FJP 2G5P 2G5T 2G63 2HHA 2I03 2I78 2IIT 2IIV 2JID 2OAG 2OGZ 2OLE 2ONC 2OPH 2OQI 2OQV 2P8S 2QJR 2QKY 2QOE 2QT9 2QTB 2RGU 2RIP 3BJM 3C43 3C45 3CCB 3CCC 3D4L 3EIO 3F8S 3G0B 3G0C 3G0D 3G0G 3H0C 3HAB 3HAC 3KWF 3KWJ 3NOX 3O95 3O9V 3OC0 3OPM 3Q0T 3Q8W 3QBJ 3SWW 3SX4 3VJM 4A5S
PDB (PDBSum)	1J2E 1N1M 1NU6 1NU8 1PFQ 1R9M 1R9N 1RWQ 1TK3 1TKR 1U8E 1W11 1WCY 1X70 2AJL 2BGN 2BGR 2BUB 2FJP 2G5P 2G5T 2G63 2HHA 2I03 2I78 2IIT 2IIV 2JID 2OAG 2OGZ 2OLE 2ONC 2OPH 2OQI 2OQV 2P8S 2QJR 2QKY 2QOE 2QT9 2QTB 2RGU 2RIP 3BJM 3C43 3C45 3CCB 3CCC 3D4L 3EIO 3F8S 3G0B 3G0C 3G0D 3G0G 3H0C 3HAB 3HAC 3KWF 3KWJ 3NOX 3O95 3O9V 3OC0 3OPM 3Q0T 3Q8W 3QBJ 3SWW 3SX4 3VJM 4A5S
PDB (IMB)	1J2E 1N1M 1NU6 1NU8 1PFQ 1R9M 1R9N 1RWQ 1TK3 1TKR 1U8E 1W11 1WCY 1X70 2AJL 2BGN 2BGR 2BUB 2FJP 2G5P 2G5T 2G63 2HHA 2I03 2I78 2IIT 2IIV 2JID 2OAG 2OGZ 2OLE 2ONC 2OPH 2OQI 2OQV 2P8S 2QJR 2QKY 2QOE 2QT9 2QTB 2RGU 2RIP 3BJM 3C43 3C45 3CCB 3CCC 3D4L 3EIO 3F8S 3G0B 3G0C 3G0D 3G0G 3H0C 3HAB 3HAC 3KWF 3KWJ 3NOX 3O95 3O9V 3OC0 3OPM 3Q0T 3Q8W 3QBJ 3SWW 3SX4 3VJM 4A5S
PDB (RSDB)	1J2E 1N1M 1NU6 1NU8 1PFQ 1R9M 1R9N 1RWQ 1TK3 1TKR 1U8E 1W11 1WCY 1X70 2AJL 2BGN 2BGR 2BUB 2FJP 2G5P 2G5T 2G63 2HHA 2I03 2I78 2IIT 2IIV 2JID 2OAG 2OGZ 2OLE 2ONC 2OPH 2OQI 2OQV 2P8S 2QJR 2QKY 2QOE 2QT9 2QTB 2RGU 2RIP 3BJM 3C43 3C45 3CCB 3CCC 3D4L 3EIO 3F8S 3G0B 3G0C 3G0D 3G0G 3H0C 3HAB 3HAC 3KWF 3KWJ 3NOX 3O95 3O9V 3OC0 3OPM 3Q0T 3Q8W 3QBJ 3SWW 3SX4 3VJM 4A5S
Human Protein Atlas	ENSG00000197635
HPRD	02187
IPI	IPI00018953 IPI00924974 IPI00924746 IPI00927124
	Protein Interaction databases
DIP (DOE-UCLA)	P27487
IntAct (EBI)	P27487
FunCoup	ENSG00000197635
REACTOME	DPP4
Protein Interaction Database	1803
BioGRID	DPP4
InParanoid	P27487
Interologous Interaction database	P27487
IntegromeDB	DPP4
	Polymorphism : SNP, mutations, diseases
	DPP4

SNP Single Nucleotide Polymorphism (NCBI)	
SNP (GeneSNP Utah)	DPP4
SNP : HGBase	DPP4
Genetic variants : HAPMAP	DPP4
Somatic Mutations in Cancer : COSMIC	DPP4
CONAN: Copy Number Analysis	DPP4
Mutations and Diseases : HGMD	DPP4
OMIM	102720
GENETests	102720
Disease Genetic Association	DPP4
Huge Navigator	DPP4 [HugePedia] DPP4 [HugeCancerGEM]
Genomic Variants	DPP4 DPP4 [DGVbeta]
snp3D : Map Gene to Disease	1803
	General knowledge
Homologs : HomoloGene	DPP4
Homology/Alignments : Family Browser (UCSC)	DPP4
Phylogenetic Trees/Animal Genes : TreeFam	DPP4
Catalytic activity : Enzyme	3.4.14.5 [Enzyme-Expasy] 3.4.14.5 [Enzyme-SRS] 3.4.14.5 [IntEnz-EBI] 3.4.14.5 [BRENDA] 3.4.14.5 [KEGG]
Chemical/Protein Interactions : CTD	1803
Chemical/Pharm GKB Gene	PA27467
Clinical trial	DPP4
Cancer Resource (Charite)	ENSG00000197635
Ontology : AmiGO	response to hypoxia protease binding regulation of T cell mediated immunity aminopeptidase activity serine-type endopeptidase activity receptor binding protein binding collagen binding endoplasmic reticulum Golgi apparatus plasma membrane proteolysis cell adhesion serine-type peptidase activity dipeptidyl-peptidase activity positive regulation of cell proliferation cell surface negative regulation of extracellular matrix disassembly integral to membrane apical plasma membrane lamellipodium endocytic vesicle lamellipodium membrane T cell costimulation regulation of cell-cell adhesion mediated by integrin T cell activation peptide binding protein homodimerization activity endothelial cell migration membrane raft intercellular canaliculus establishment of localization extracellular vesicular exosome invadopodium membrane
Ontology : EGO-EBI	response to hypoxia protease binding regulation of T cell mediated immunity aminopeptidase activity serine-type endopeptidase activity receptor binding protein binding collagen binding endoplasmic reticulum Golgi apparatus plasma membrane proteolysis cell adhesion serine-type peptidase activity dipeptidyl-peptidase activity positive regulation of cell proliferation cell surface negative regulation of extracellular matrix disassembly integral to membrane apical plasma membrane lamellipodium endocytic vesicle lamellipodium membrane T cell costimulation regulation of cell-cell adhesion mediated by integrin T cell activation peptide binding protein homodimerization activity endothelial cell migration membrane raft intercellular canaliculus establishment of localization extracellular vesicular exosome invadopodium membrane
	Other databases
	Probes
	Litterature
PubMed	239 Pubmed reference(s) in Entrez
PubGene	DPP4
iHOP	DPP4

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IMMUNOLOGY ORIGINAL ARTICLE

CD26-mediated co-stimulation in human CD8$^+$ T cells provokes effector function via pro-inflammatory cytokine production

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Summary

CD26 is an activation marker of human CD4$^+$ T cells, and is associated with T-cell signal transduction processes as a co-stimulatory molecule. We have previously demonstrated that high CD26 cell surface expression on CD4$^+$ T cells is correlated with the production of T helper type 1 cytokines, whereas CD26$^+$ T helper cells stimulate antibody synthesis in B cells. Although the cellular and molecular mechanisms involved in CD26-mediated CD4$^+$ T-cell activation have been extensively evaluated by our group and others, the role of CD26 in CD8$^+$ T cells has not been clearly elucidated. In the present study, we examine the effector function of CD8$^+$ T cells via CD26-mediated co-stimulation in comparison with CD28-mediated co-stimulation. We found that CD26^{high} CD8$^+$ T cells belong to the early effector memory T-cell subset, and that CD26-mediated co-stimulation of CD8$^+$ T cells exerts a cytotoxic effect preferentially via granzyme B, tumour necrosis factor-α, interferon-γ and Fas ligand. The effector function associated with CD26-mediated co-stimulation is enhanced compared with that obtained through CD28-mediated co-stimulation, suggesting that the CD26 co-stimulation pathway in CD8$^+$ T cells is distinct from the CD28 co-stimulation pathway. Targeting CD26 in CD8$^+$ T cells therefore has the potential to be useful in studies of immune responses to new vaccine candidates as well as innovative therapy for immune-mediated diseases.

Keywords: CD26/dipeptidyl peptidase 4; CD8; cytotoxic T-lymphocyte effect; effector memory; granzyme B.

Introduction

In addition to being a marker of T-cell activation, CD26 is associated with T-cell signal transduction processes as a co-stimulatory molecule, and the enzymatic activity of CD26 appears to play an important role in enhancing cellular responses to external stimuli.[1] Whereas CD26 expression is increased following activation of resting T cells, CD4$^+$ CD26^{high} T cells respond maximally to recall antigens such as tetanus toxoid.[2] Moreover, cross-linking of CD26 and CD3 with solid-phase immobilized monoclonal antibodies (mAbs) can induce T-cell co-stimulation and interleukin-2 (IL-2) production by CD26$^+$ T cells.[1] High CD26 cell surface expression in CD4$^+$ T cells is correlated with the production of T helper type 1 (Th1) cytokines and high migratory activity, whereas CD26$^+$ T helper cells stimulate antibody synthesis in B cells.[1] Recently, we have demonstrated that caveolin-1 is a co-stimulatory ligand for CD26 in CD4$^+$ T cells, and that CD26 on activated memory CD4$^+$ T cells interacts with caveolin-1 on tetanus toxoid-loaded monocytes.[3,4] Moreover, following CD26–caveolin-1 interaction on tetanus toxoid-loaded monocytes, caveolin-1 is phosphorylated, with linkage to nuclear factor-κB activation, followed by up-regulation of CD86, a ligand for CD28.[5,6] Taking into account the data that effector T cells in inflamed lesions express high levels of CD26, it is conceivable that CD4$^+$ CD26$^+$ T cells play an important role in the inflammatory process.

It has been recently reported that influenza-specific CD8 'memory' T cells express high levels of CD26, whereas CD8$^+$ T cells specific for chronically infecting viruses such as cytomegalovirus, Epstein–Barr virus and HIV do not express CD26.[7] This suggests that high expression of CD26 on CD8$^+$ T cells may offer a specific marker of successful memory development. More recently, it has been shown that terminally differentiated effector memory cells (CD45RA$^+$ CCR7$^-$) among

$CD8^+$ $CD26^+$ T cells are markedly increased in patients with type 1 diabetes, suggesting that the pathogenesis of type 1 diabetes might be associated with life-long stimulation by protracted antigen exposure or a homeostatic defect in the regulation/contraction of immune responses.[8]

Although the above studies analysed the phenotype of CD26-expressing $CD8^+$ T cells, more extensive research is required to fully characterize the phenotype of $CD8^+$ $CD26^+$ T cells and cellular function of $CD8^+$ T cells via CD26-mediated co-stimulation. In the present study, to explore the immunological role of CD26-expressing $CD8^+$ T cells, we examined the effector function of $CD8^+$ T cells via CD26-mediated co-stimulation in comparison with CD28-mediated co-stimulation. We found that $CD26^{high}$ $CD8^+$ T cells are among the subset of early effector memory T cells, and that CD26-mediated co-stimulation in $CD8^+$ T cells exerts cytotoxic effects preferentially via granzyme B, tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and Fas ligand (FasL). The effector function associated with CD26-mediated co-stimulation is enhanced in comparison with that obtained with CD28-mediated co-stimulation.

Materials and methods

Cells and antibodies

Human $CD3^+$ T cells or $CD8^+$ T cells were purified from peripheral blood mononuclear cells (PBMC) of healthy adult volunteers after their documented informed consent was obtained. This study has been performed according to the Declaration of Helsinki, and the process involved has also been approved by the institutional review board. For purification, a MACS human Pan T-cell Isolation kit or MACS human $CD8^+$ T-cell Isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) was used. Non-$CD8^+$ T cells, namely $CD4^+$ T cells, monocytes, neutrophils, eosinophils, B cells, stem cells, dendritic cells, natural killer cells, granulocytes, $\gamma\delta$ T cells or erythroid cells were specifically depleted by using antibodies against CD4, CD15, CD16, CD19, CD34, CD36, CD56, CD123, T-cell recptor-$\gamma\delta$ and CD235a. Purity of $CD3^+$ T cells or $CD8^+$ T cells was $\geq$ 99% or $\geq$ 95%, respectively, as confirmed by FACSCalibur (BD Biosciences, San Jose, CA). No cytotoxic lymphocytes other than $CD8^+$ T cells, such as natural killer cells, were detected in the purified $CD8^+$ T-cell fraction. For cell stimulation, anti-CD3 mAb (OKT3), anti-CD28 mAb (4B10) and anti-CD26 mAb (1F7) were developed in our laboratory. Other antibodies used for flow cytometry were purchased from BD Biosciences. In experiments involving CD26-positive or negative selection, purified $CD8^+$ T cells were further separated into positively or negatively selected fractions using anti-CD26 mAb (5F8) and anti-mouse IgG_1-conjugated magnetic beads (Miltenyi Biotec). 5F8 was developed in our laboratory, and exerts no effect on $CD26^+$ T-cell function, as well as having no cross-reaction with 1F7.[9]

Detection of intracellular cytotoxic granules

Purified $CD8^+$ T cells (1×10^5) were cultured in serum-free AIM-V medium (Invitrogen, Carlsbad, CA) in 96-well flat-bottom plates (Costar, Corning Incorporated, Corning, NY), with stimulatory mAbs being bound in the wells beforehand at the following concentrations; 0·5 μg/ml of OKT3, and/or 2, 5, 10, 20 or 50 μg/ml of 4B10 or 1F7. Cells were cultured in a 5% CO_2 and 100% humidified incubator at 37° for the indicated time intervals, and cells were then prepared for analysis of intracellular perforin (PRF), granzyme A (GzmA) or granzyme B (GzmB) using a BD Cytofix/Cytoperm Plus Fixation/Permeabilization kit (BD Biosciences). The data obtained were analysed with FLOWJO software (Tree Star, Inc., Ashland, OR).

Measurement of cytokines

Purified $CD8^+$ T cells (1×10^5) were incubated with plate-bound OKT3 (0·5 μg/ml) and/or 4B10 (5 μg/ml) or 1F7 (5 μg/ml) in 96-well flat-bottom plates for 48, 72, 96 or 120 hr. After incubation, supernatants were collected and cytokine concentrations were examined using ELISA. BD OptEIA kits for human IL-2, TNF-α or IFN-γ were purchased from BD Biosciences, and a FasLigand/TNFSF6 DuoSet for soluble FasL (sFasL) was purchased from R&D Systems (Minneapolis, MN).

Quantitative real-time reverse transcription-PCR assay

Purified $CD8^+$ T cells (6×10^5) were incubated with plate-bound OKT3 (0·5 μg/ml) and/or 4B10 (5 μg/ml) or 1F7 (5 μg/ml) in 24-well flat-bottom plates (Costar) for 12, 24, 48 or 72 hr. After incubation, cells were collected and total RNA was extracted using an RNeasy Micro kit according to the manufacturer's instructions (QIAGEN, Valencia, CA). Complementary DNA was produced using a PrimeScript II 1st strand cDNA Synthesis kit (TaKaRa Bio, Shiga, Japan) with oligo-dT primer. Quantification of mRNA was performed using the 7500 Real-Time PCR System and SYBR Select Master Mix (Applied Biosystems, Foster City, CA). The data obtained were analysed with 7500 System SDS Software (Applied Biosystems), being normalized to hypoxanthine phosphoribosyltransferase (HPRT) expression. The PCR was performed using the following primers: TNF-α forward primer, 5′-TCAG CCTCTTCTCCTTCCTG-3′; reverse primer, 5′-TTTGCTA CAACATGGGCTACA-3′; IFN-γ forward primer, 5′-GTG TGGAGACCATCAAGGAAG-3′; reverse primer, 5′-ATG TATTGCTTTGCGTTGGA-3′; FasL forward primer, 5′-TG GGGATGTTTCAGCTCTTC-3′; reverse primer, 5′-TGGA

CCTTGAGTTGGACTTG-3′; IL-2 forward primer, 5′-AG AAGGCCACAGAACTGAAAC-3′; reverse primer, 5′-GCT GTCTCATCAGCATATTCAC-3′; HPRT1 forward primer, 5′-CAGTCAACAGGGGACATAAAAG-3′; reverse primer, 5′-CCTGACCAAGGAAAGCAAAG-3′.

Mixed lymphocyte reaction assay

Purified $CD8^+$ T cells (1×10^6) were incubated with plate-bound OKT3 (0·5 μg/ml) and/or 4B10 (5 μg/ml) or 1F7 (5 μg/ml) in 24-well flat-bottom plates for 72 hr. The incubated effector $CD8^+$ T cells were co-cultured in 96-well V-bottom plates (NUNC, Roskilde, Denmark) with AIM-V medium admixed with carboxyfluorescein succinimidyl ester (CFSE) -labelled U937 cells (1×10^5) as target cells. CFSE labelling of U937 cells was conducted using a Vybrant CFDASE Cell Tracer kit (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. The number of target cells was fixed at 1×10^5/well in the present experiments, and effector to target (E : T) cell ratios were 1 : 1, 2 : 1, or 4 : 1. For the inhibition assays, Granzyme B Inhibitor 1 (200 μM; Calbiochem, San Diego, CA) or neutralizing anti-FasL mAb (10 μg/ml; BD Biosciences) was added to the medium for incubation at an E : T ratio of 2 : 1. After 16 hr of co-culture, cells were harvested and stained with an Annexin V-PE Apoptosis Detection kit I (BD Biosciences). CFSE-positive cells (U937 target cells) were gated in and Annexin V-positive and 7-amino-actinomycin D (7-AAD) -positive cells were detected using FACSCalibur, with data being analysed with FlowJo software.

Statistics

The paired Student's *t*-test (two-tailed) was used for the comparison of group values. The assay was performed in triplicate wells, and data are presented as mean ± SE of triplicate samples of independent experiments. Significance was analysed using MS-EXCEL (Microsoft, Redmond, WA), and values of $P < 0{\cdot}01$ were considered significant and indicated in the corresponding figures and figure legends.

Results

Human $CD26^{high}$ $CD8^+$ T cells belong to an early effector memory subset

To characterize the phenotype of CD26-expressing $CD8^+$ T cells, we first used flow cytometry to conduct cell surface marker analysis of $CD8^+$ T cells derived from human PBMC. As shown in Fig. 1(a), $CD8^+$ T cells were divided into four subsets of $CD26^{high}$ $CD28^+$, $CD26^{int}$ $CD28^+$, $CD26^-$ $CD28^+$ and $CD26^-$ $CD28^-$ populations. For further examination, we performed multi-colour analysis by flow cytometry of these four populations. As shown in Fig. 1(b), $CD26^{high}$ (exclusively $CD28^+$) $CD8^+$ T cells were enriched in the $CD45RA^-$ $CCR7^-$ population (82·3 ± 1·7%, $n = 5$) (Fig. 1b-i), whereas $CD26^{int}$ (exclusively $CD28^+$) $CD8^+$ T cells were enriched in the $CD45RA^+$ $CCR7^+$ population (78·2 ± 2·8%, $n = 5$) (Fig. 1b-ii). Moreover, $CD26^-$ $CD28^+$ or $CD26^-$ $CD28^-$ $CD8^+$ T cells were enriched in $CD45RA^-$ and $CD45RA^+$ $CCR7^-$ (62·5 ± 5·9% and 24·7 ± 2·0%,

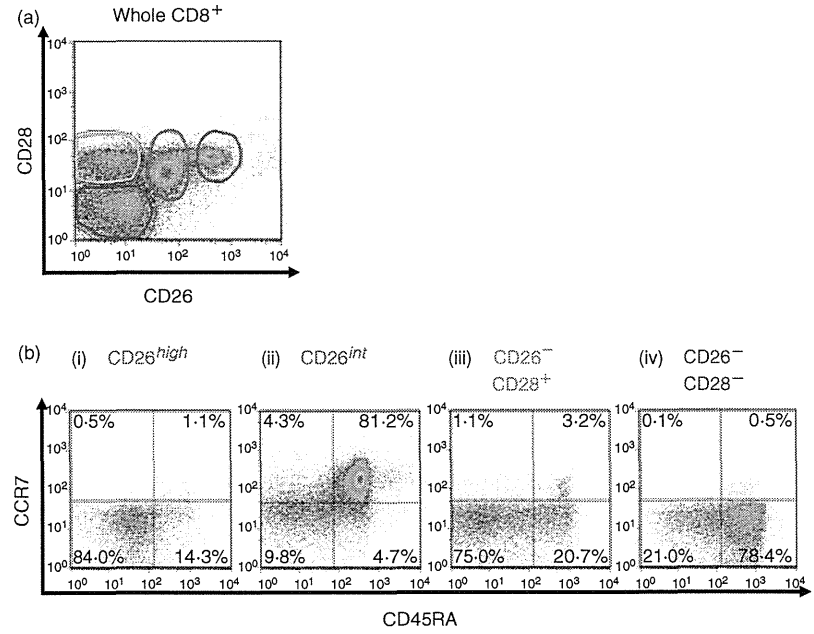

Figure 1. $CD26^{high}$ $CD8^+$ T cells are an effector memory subset, as characterized by cell surface marker analysis. Purified $CD8^+$ T cells from peripheral blood mononuclear cells were stained with CD26, CD28, CD45RA, and CCR7 monoclonal antibodies. Cells were then analysed by multi-colour flow cytometry. (a) Two-dimensional (2D) dot blot of CD26 or CD28 staining gated for $CD8^+$ T cells, showing a representative plot of five independent donors. (b) 2D-dot blot of CD45RA or CCR7 staining gated for $CD26^{high}$, $CD26^{int}$, $CD26^-$ $CD28^+$ or $CD26^-$ $CD28^-$ cells among $CD8^+$ T cells, showing a representative plot of five independent donors.

respectively, $n = 4$) (Fig. 1b-iii), or CD45RA$^+$ CCR7$^-$ population (71·7 ± 4·9%, $n = 4$) (Fig. 1b-iv), respectively. These data indicate that CD26^{high} CD8$^+$ T cells are effector memory (EM) cells and that CD26^{int} CD8$^+$ T cells are naive cells.[10]

For further confirmation, we analysed the expression pattern of cytotoxic granules in CD8$^+$ T cells through flow cytometry. Whole CD8$^+$ T cells of human PBMC showed a triphasic pattern of expression for PRF (Fig. 2a-i), and a biphasic pattern of expression for GzmA (Fig. 2a-ii) or GzmB (Fig. 2a-iii), suggesting the presence of heterogeneous populations. Compared with the analysis of cell surface markers of CD8$^+$ T cells, as shown in Fig. 1, we analysed expression levels of PRF, GzmA or GzmB among CD26^{high} CD8$^+$ (exclusively CD28$^+$), CD26^{int} CD8$^+$ (exclusively CD28$^+$), CD26$^-$ CD28$^+$ CD8$^+$, or CD26$^-$ CD28$^-$ CD8$^+$ subsets. CD26^{high} CD8$^+$ T cells were PRF^{int} GzmA$^+$ GzmB$^{low/-}$ (Fig. 2b-i–iii), whereas CD26^{int} CD8$^+$ T cells were PRF$^-$ GzmA$^-$ GzmB$^{low/-}$ (Fig. 2c-i–iii). These data indicate that CD26^{high} CD8$^+$ T cells are an early EM subset and that CD26^{int} CD8$^+$ T cells are a naive or central memory subset.[11] On the other hand, CD26$^-$ CD28$^+$ CD8$^+$ T cells were composed of PRF^{int} or PRF$^-$, GzmA$^+$ or GzmA$^-$, and GzmB^{high} or GzmB$^{low/-}$ populations (Fig. 2d-i–iii). These data indicate that CD26$^-$ CD28$^+$ CD8$^+$ T cells are collective populations rather than a terminally differentiated effector memory (TEMRA) subset.[11] In contrast, CD26$^-$ CD28$^-$ CD8$^+$ T cells contained PRF^{high} or PRF^{int}, GzmA$^+$ and GzmB^{high} populations (Fig. 2e-i–iii), indicative of TEMRA and late EM subsets.[11]

Taken together, these data indicate that CD26^{high} CD8$^+$ T cells are an early EM subset and CD26^{int} CD8$^+$ T cells are a naive subset, whereas CD28$^+$ CD8$^+$ T cells are composed of heterogeneous subsets. Therefore, these findings strongly suggest that CD26$^+$ CD8$^+$ T cells may have an effector function that is different from that of the CD28$^+$ subset of CD8$^+$ T cells.

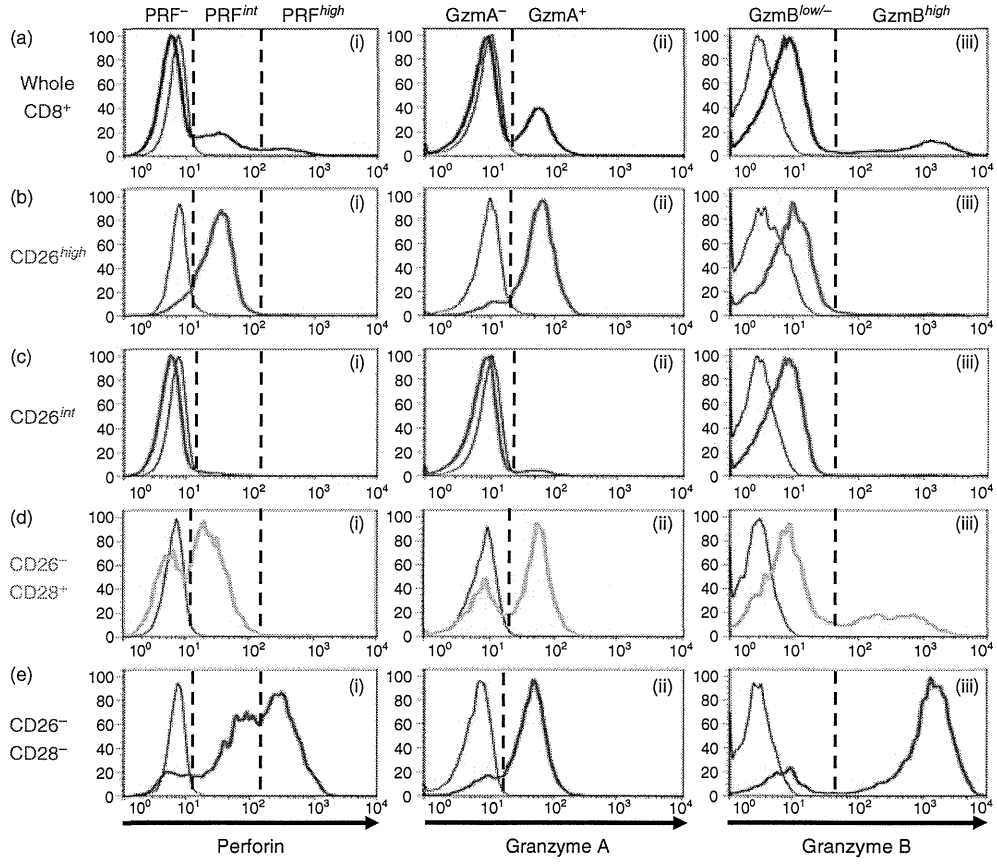

Figure 2. CD26^{high} CD8$^+$ T cells are an early effector memory subset, as characterized by analysis of intracellular cytotoxic granules. Purified CD8$^+$ T cells from peripheral blood mononuclear cells were stained with CD26, CD28, perforin (PRF), granzyme A (GzmA) and granzyme B (GzmB) monoclonal antibodies, and analysed by flow cytometry. Following gating for whole CD8$^+$ population (brown lines in a), CD8$^+$ CD26^{high} population (red lines in b), CD8$^+$ CD26^{int} population (green lines in c), CD8$^+$ CD26$^-$ CD28$^+$ population (blue lines in d), or CD8$^+$ CD26$^-$ CD28$^-$ population (purple lines in e), each subset was analysed for the expression of PRF, GzmA or GzmB. The data are shown as histograms of PRF (i), GzmA (ii), or GzmB (iii) intensity among each subset, representative of eight independent donors. The black lines in each histogram show the data of isotype control.

CD26-mediated co-stimulation in CD8$^+$ T cells preferentially enhances excretion of GzmB as compared with CD28-mediated co-stimulation

The data above suggest that CD26$^+$ CD8$^+$ T cells have a different effector role from CD28$^+$ CD8$^+$ T cells. To examine the cellular function associated with each co-stimulatory effect, we performed co-stimulation studies using anti-CD3 and/or anti-CD26 or anti-CD28 mAbs. For this purpose, we first performed a cell proliferation assay. As shown in the Supplementary data section, neither whole T cells (pan-T cells) of human PBMC nor purified CD8$^+$ T cells exhibited proliferative activity following stimulation with anti-CD3 mAb alone (see Supplementary material, Fig. S1), whereas both pan-T cells and CD8$^+$ T cells showed equal enhancement of proliferation by anti-CD3 plus anti-CD28 or anti-CD3 plus anti-CD26 co-stimulation (Fig. S1a,b). These data indicate that CD8$^+$ T cells exhibit proliferative activity through either CD26-mediated or CD28-mediated co-stimulation. We next examined production of cytotoxic granules or cytokines in the presence of CD26-mediated or CD28-mediated co-stimulation. For this purpose, we evaluated the expression levels of PRF, GzmA or GzmB in CD8$^+$ T cells. As shown in Fig. 3(a), expression of GzmB in CD8$^+$ T cells was increased following CD26-mediated co-stimulation compared with CD28-mediated co-stimulation at any stimulation intensity (Fig. 3a). Moreover, time–course analysis showed that GzmB expression was greater over the tested time intervals after CD26-mediated co-stimulation than after CD28-mediated co-stimulation (Fig. 3b). In contrast, no difference in the expression of PRF or GzmA was observed following CD26-mediated co-stimulation or CD28-mediated co-stimulation (data not shown).

To confirm the differences in effector function associated with CD26-mediated co-stimulation in CD26^{high} CD8$^+$ T cells from that of CD26^{int} CD8$^+$ T cells, we examined the expression of GzmB in CD8$^+$ T cells following purification by anti-CD26 mAb and anti-mouse IgG$_1$ microbeads. As shown in the Supplementary material, Fig. S2, both CD26^{high} CD8$^+$ and CD26^{int} CD8$^+$ cells were selected in the positive fraction after purification (Fig. S2b), whereas CD26^{int} CD8$^+$ and CD26$^-$ CD8$^+$ cells were contained in the flow-through (negative fraction) (Fig. S2c). Using each positive or

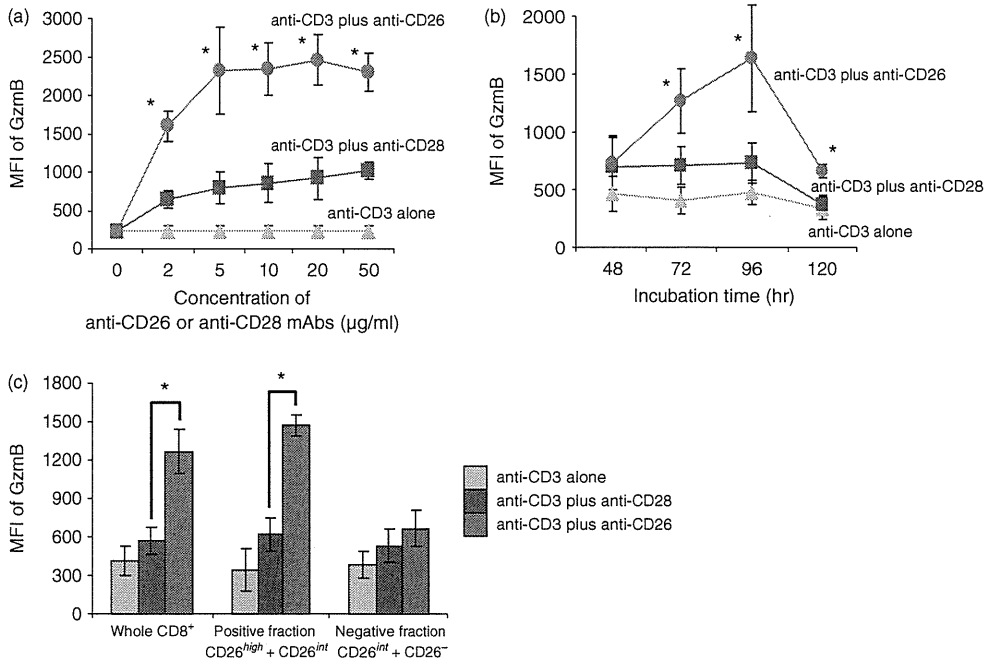

Figure 3. CD26-mediated co-stimulation of CD8$^+$ T cells induces greater Granzyme B expression than CD28-mediated co-stimulation. (a) Purified CD8$^+$ T cells were stimulated with anti-CD3 monoclonal antibodies (mAb) alone, anti-CD3 plus anti-CD28 mAbs, or anti-CD3 plus anti-CD26 mAbs at the indicated concentrations for 72 hr. (b) Purified CD8$^+$ T cells were stimulated with anti-CD3 mAb alone, anti-CD3 plus anti-CD28 mAbs (5 μg/ml), or anti-CD3 plus anti-CD26 mAbs (5 μg/ml) for the indicated time. (c) Purified CD8$^+$ T cells were separated using anti-CD26 mAb (5F8) and anti-mouse IgG$_1$-conjugated magnetic beads as described in the Materials and methods. Whole CD8$^+$ T cells, positively selected cells (positive fraction) or negatively selected cells (negative fraction) were stimulated and incubated by the same method as shown in (b) for 72 hr. Intracellular granzyme B (GzmB) was detected by flow cytometry. Data are shown as mean ± SE of mean fluorescence intensity (MFI) of GzmB from two independent donors (a) and five independent donors (b,c), comparing MFI in anti-CD3 plus anti-CD26 to that in anti-CD3 plus anti-CD28 (*$P < 0{\cdot}01$).

negative fraction, expression of GzmB following CD26 or CD28 co-stimulation was analysed. As shown in Fig. 3(c), GzmB was equally expressed in cells of the negative fraction ($CD26^{int}$ $CD8^+$ and $CD26^-$ $CD8^+$ cells) either by CD26 co-stimulation or by CD28 co-stimulation. On the other hand, in the positive fraction (mixture of $CD26^{high}$ $CD8^+$ and $CD26^{int}$ $CD8^+$ cells), GzmB expression was much more enhanced by CD26-mediated co-stimulation than by CD28-mediated co-stimulation (* in Fig. 3c). These observations strongly suggest that the $CD26^{high}$ $CD8^+$ subset, but not the $CD26^{int}$ $CD8^+$ subset, is essential to induce distinctive CD26-mediated effector function from CD28-mediated co-stimulation.

In addition to increased GzmB expression, production of TNF-α, IFN-γ and sFasL in $CD8^+$ T cells was greater following CD26 co-stimulation than CD28 co-stimulation (Fig. 4a-i–iii). In contrast with these inflammatory cytokines, production of IL-2 by $CD8^+$ T cells was apparently less following CD26 co-stimulation than the level seen with CD28 co-stimulation (Fig. 4a-iv). The same effect was also found on the cytokine production over a wide range of anti-CD26 and anti-CD28 mAbs tested as well as the induction of GzmB expression (data not shown). To confirm the above results regarding cytokine production, we conducted real-time RT-PCR. As shown in Fig. 4(b), mRNA expression of TNF-α, IFN-γ or IL-2 (but not FasL) was significantly up-regulated following CD28

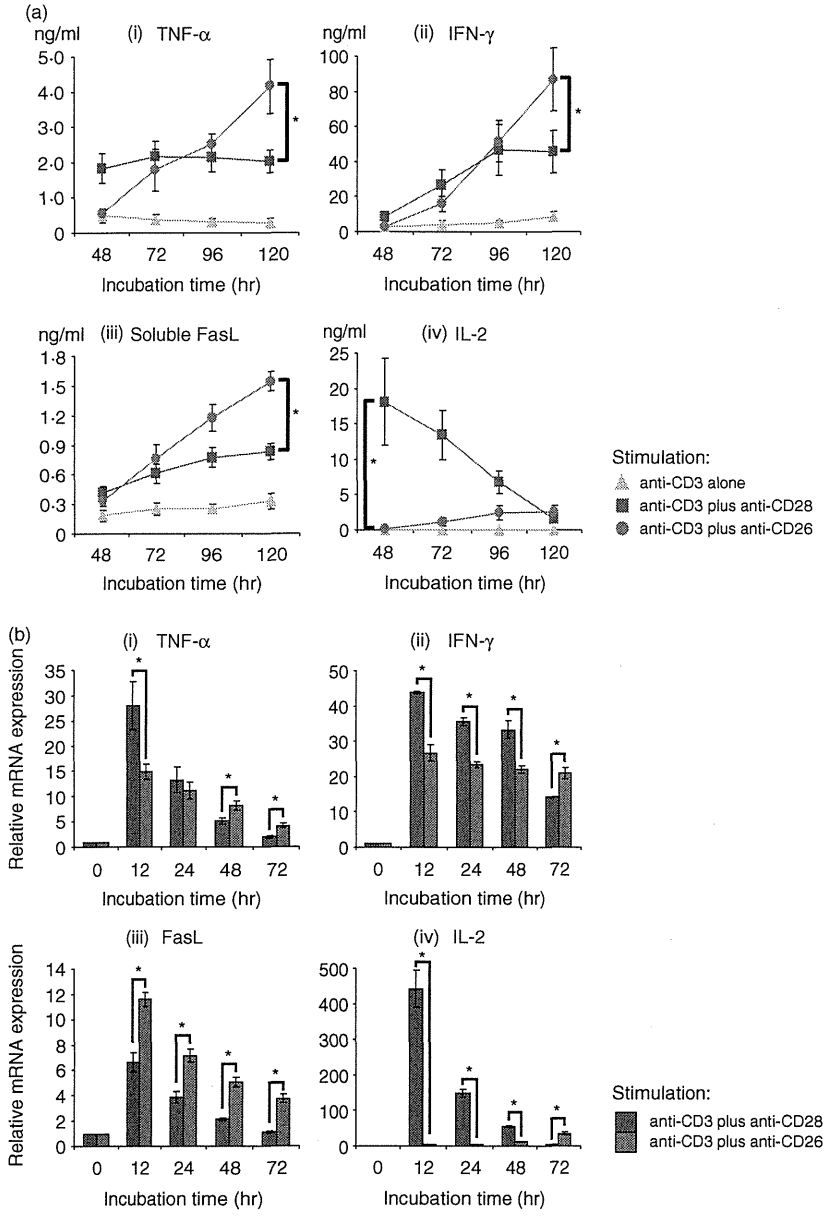

Figure 4. CD26-mediated co-stimulation of $CD8^+$ T cells induces greater levels of tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and soluble Fas ligand (sFasL) than CD28-mediated co-stimulation. Purified $CD8^+$ T cells were stimulated with anti-CD3 monoclonal antibody (mAb) alone, anti-CD3 plus anti-CD28 mAbs, or anti-CD3 plus anti-CD26 mAbs for the indicated times. (a) Concentrations of TNF-α (i), IFN-γ (ii), sFasL (iii) and interleukin-2 (IL-2) (iv) were examined by ELISA. (b) mRNA expression of TNF-α (i), IFN-γ (ii), FasL (iii) and IL-2 (iv) was quantified by real-time RT-PCR. Each expression was normalized to HPRT1 and relative expression levels compared with resting $CD8^+$ T cells (0 hr) were shown. Data are shown as mean ± SE of triplicate wells from six independent donors (a) and shown as mean ± SD of triplicate samples (b), comparing values in anti-CD3 plus anti-CD26 to that in anti-CD3 plus anti-CD28 (*$P < 0{\cdot}01$).

co-stimulation compared with CD26 co-stimulation at the 12 hr incubation time period. Meanwhile, mRNA expression levels of all the cytokines tested were higher after 72 hr of CD26 co-stimulation than CD28 co-stimulation (Fig. 4b-i–iv). These results correlated with the kinetics of cytokine production shown in Fig. 4(a). Taken together, these data suggest that CD26 co-stimulation results in enhanced $CD8^+$ T-cell effector function by increasing production of GzmB, TNF-α, IFN-γ or sFasL, compared with CD28 co-stimulation.

CD26-mediated co-stimulation of $CD8^+$ T cells preferentially enhances cytotoxic effect compared with CD28-mediated co-stimulation

Finally, we conducted cytotoxic assays by mixed lymphocyte reaction (MLR) in the presence of CD26-mediated or CD28-mediated co-stimulation. For this purpose, CFSE-labelled U937 cells were co-cultured with activated $CD8^+$ T cells, and then cells were harvested and stained with Annexin V-PE and 7-AAD. CFSE-positive cells (U937) as target cells were gated in to detect Annexin V^+ $7\text{-}AAD^+$ dead cells (see Supplementary material, Fig. S3a). As shown in Fig. 5, higher levels of Annexin V^+ $7\text{-}AAD^+$ cells were found by CD26-mediated cytotoxic effect than by CD28-mediated effect (* in Fig. 5). This cytotoxic effect of $CD8^+$ T cells induced by CD26-mediated co-stimulation was enhanced with increasing levels of effector cells in a dose-dependent manner (Fig. 5). Moreover, this cytotoxic effect via CD26-mediated co-stimulation was significantly decreased by GzmB inhibitor or anti-FasL neutralizing antibody (Fig. S3b). These data strongly suggest that CD26-mediated co-stimulation is sufficient to induce cytotoxic effector function in $CD8^+$ T cells via GzmB and/or FasL and that the CD26 pathway of co-stimulation is distinctive from the CD28 pathway.

Discussion

In the present study, we demonstrate that $CD26^{high}$ $CD8^+$ T cells belong to an early EM subset and that the cytotoxic effect of human $CD8^+$ T cells is exerted in part by CD26-mediated co-stimulation of $CD26^{high}$ $CD8^+$ T cells, resulting in enhanced expression of GzmB, TNF-α, IFN-γ and sFasL.[12–14]

Analysis of cell surface markers and intracellular cytotoxic granules indicates that $CD26^{high}$ $CD8^+$ T cells exclusively express CD28 and belong to an early EM subset (Figs 1 and 2). In fact, CD26-mediated co-stimulation of $CD26^{high}$ $CD8^+$ T cells induced increased expression levels of GzmB, TNF-α, IFN-γ and sFasL, as well as enhanced cytotoxic effect (Figs 3, 4 and 5). Of note is that CD26-mediated co-stimulation resulted in greater effector function than that induced by CD28-mediated co-stimulation. These findings strongly suggest that the CD26 co-stimulation pathway in $CD8^+$ T cells resulting in enhanced cytotoxic effect is distinct from the CD28 co-stimulation pathway. In contrast to $CD26^{high}$ $CD8^+$ T cells, $CD26^{int}$ $CD8^+$ T cells belong to the naive subset (Figs 1 and 2). Moreover, co-stimulation through either the CD26 or CD28 pathway did not result in differences in effector function of $CD26^{int}$ $CD8^+$ T cells (Fig. 3). Purification of $CD26^{high}$ $CD8^+$ and $CD26^{int}$ $CD8^+$ T cells by cell sorting may be required for more precise analyses of effector function of these two subsets instead of using the MACS separation system.

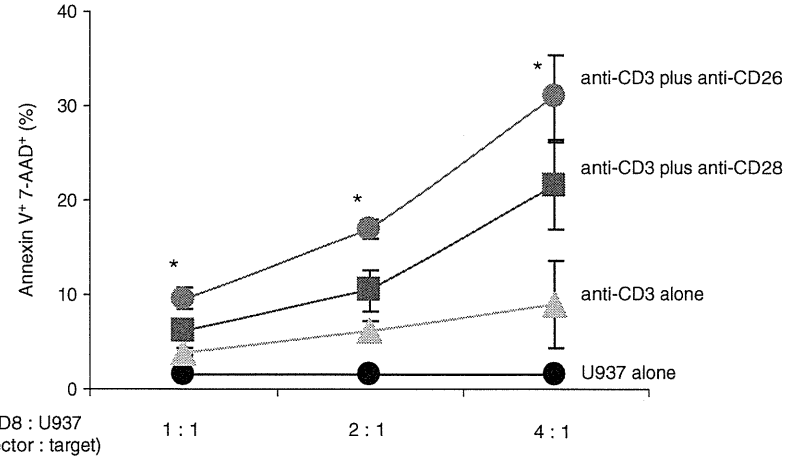

Figure 5. CD26-mediated co-stimulation of $CD8^+$ T cells results in greater cytotoxic effect than CD28-mediated co-stimulation. Stimulated $CD8^+$ T cells and CFSE-labelled U937 cells were used as effector and target cells, respectively. Mixed lymphocyte reaction assay using these effector and target cells was conducted as described in Materials and methods. Data are shown as mean ± SE of % positive cells stained with Annexin V and 7-AAD among CFSE-positive cells (U937 target cells) from four independent donors, comparing percentage of positive cells in anti-CD3 plus anti-CD26 to that in anti-CD3 plus anti-CD28 (*$P < 0{\cdot}01$).

The expression pattern of cytotoxic granules or cytokines in $CD8^+$ T cells induced by CD26-mediated co-stimulation was clearly distinct from that induced by CD28-mediated co-stimulation. As shown in Figs 3 and 4, GzmB (Fig. 3), TNF-α (Fig. 4a-i), IFN-γ (Fig. 4a-ii) and sFasL (Fig. 4a-iii) levels were higher in CD26-stimulated $CD8^+$ T cells than in CD28-stimulated $CD8^+$ T cells. Moreover, CD26-mediated co-stimulation resulted in greater cytotoxic effect as analysed by MLR assays than CD28-mediated co-stimulation (Fig. 5). These results may be partly because the $CD28^+$ subset in $CD8^+$ T cells is composed of heterogeneous cell populations, whereas the $CD26^+$ $CD8^+$ T cells only consist of an early EM ($CD26^{high}$) and a naive ($CD26^{int}$) subset (Figs 1 and 2). Besides differences in cytotoxic effect, IL-2 production in $CD8^+$ T cells was also different in CD26-mediated co-stimulation compared with CD28-mediated co-stimulation (Fig. 4a-iv). Lower production of IL-2 induced by CD26-mediated co-stimulation may produce lower proliferative activity in early phase as shown in Fig. S1 (* at 72 hr). Our present data therefore support the notion that the signalling pathway via CD26 is distinct from signalling induced through the CD28 molecule, as we have previously demonstrated in human $CD4^+$ T cells,[15,16] although the exact mechanisms involved remain to be elucidated.

Taken together, our present findings strongly suggest that $CD26^{high}$ $CD8^+$ T cells have a distinctive effector function in humans, and that the CD26-mediated co-stimulation pathway is different from that of CD28. In this regard, $CD26^{high}$ $CD8^+$ T cells may have a key role in acquired immune reactions such as antigen-specific host defence against infection or cancer, and the pathophysiology of autoimmune diseases or transplantation-related immune disorders such as graft-versus-host disease or graft rejection. Therefore, targeting $CD26^{high}$ $CD8^+$ T cells has the potential to be useful in studies of immune responses to new vaccine candidates as well as innovative therapies for immune-mediated diseases.

Acknowledgements

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Conflicts of interests

None.

Disclosure

None.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. CD26-mediated co-stimulation or CD28-mediated co-stimulation equally enhances proliferation of $CD8^+$ T cells as well as $CD3^+$ T cells.

Figure S2. CD26 expression of $CD8^+$ T cells following isolation by anti-human CD26 monoclonal antibody-conjugated magnetic beads.

Figure S3. CD26-mediated co-stimulation enhances cytotoxic function of $CD8^+$ T cells as analysed by mixed lymphocyte reaction assays.

CD9 expression as a favorable prognostic marker for patients with malignant mesothelioma

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Abstract. CD9 is involved in cell growth, adhesion and motility and its expression is reported to be of prognostic significance in various types of human malignancies. We found increased cell migration in the mesothelioma cell lines MSTO-211H and TUM1 following *in vitro* shRNA-mediated knockdown of CD9 expression. We investigated CD9 expression in 112 malignant pleural mesotheliomas. CD9 expression was observed in 62 of 71 epithelioid, 13 of 20 biphasic and only 1 of 21 sarcomatoid mesotheliomas. Among the epithelioid mesotheliomas (EMs), CD9 expression was observed in all of the 33 cases with a differentiated type (EM-D) and in 29 of the 38 cases with a less-differentiated type (EM-LD). Patients with CD9 expression showed higher 1- and 2-year survival rates (63 and 25%) compared to the patients without CD9 expression (39 and 11%). Univariate analysis revealed that patients with CD9 expression demonstrated a more favorable survival (P=0.0025) along with other clinicopathological factors, including age younger than 60 years, IMIG stage I-II, epithelioid histology, EM-D and patients who underwent extrapleural pneumonectomy or received chemotherapy. Multivariate analysis identified CD9 expression as an independent prognostic factor with a hazard ratio (HR) of 1.99 in the analysis of all mesotheliomas (P=0.0261) and an HR of 2.60 in the analysis of EMs (P=0.0376). CD9 expression is an independent favorable prognostic marker of malignant mesothelioma.

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Key words: CD9-shRNA, migration, CD9 immunohistochemistry, mesothelioma, survival

Introduction

CD9, a 24- to 27-kDa cell surface glycoprotein, is a member of the tetraspanin superfamily. It is expressed in numerous normal tissues and plays a critical role in various types of cell processes, such as cell adhesion, motility and various signaling pathways involving integrins. In malignancies, its expression usually suppresses tumor progression and metastasis by inhibition of tumor proliferation and survival (1,2). Although converse functions have also been reported in certain tumors, downregulation of CD9 correlates well with tumor progression or metastasis in bladder, breast, lung and colon cancers (2). An *in vivo* study using administration of the CD9 antibody to mice bearing human gastric cancer xenografts showed inhibition of tumor progression via anti-proliferative, pro-apoptotic and anti-angiogenetic effects (3), suggesting its potential for the molecular-targeted therapy of human malignancies. Moreover, we previously identified CD9, along with side population, CD24 and CD26 cells, as a cancer stem cell marker of mesothelioma, thus demonstrating its potential for cancer stem cell-targeted therapy in the future (4).

Malignant mesothelioma is an aggressive cancer with few patients surviving beyond 2 years following diagnosis. The median survival of patients without any treatment barely exceeds 1 year. A large population-based study reported 6-month, 1-year and 5-year overall survival rates of 55, 33 and 5% in mesothelioma (5). In Japanese patients, the median survival of mesothelioma has been reported to be 9-10 months from the date of diagnosis (6,7).

The clinical predictors for poor survival in patients with mesothelioma are reported to include sarcomatoid histology, older age, advanced IMIG stage, patients without palliative surgery or chemotherapy. Other biological prognostic factors such as serum and tumor EGFR expression (8,9), pleural effusion VEGF level (10), angiopoietin-1 expression (11), ER-β expression (12), methylation profile (6,13) and miRNA signatures (14) have also been reported. In this study, we identified CD9 as an independent predictor of survival, and loss of expression showed biological aggressive behavior in mesothelioma cells.

Materials and methods

Cell line. Mesothelioma cell lines, MSTO-211H [derived from biphasic mesothelioma (BM)] and TUM1 (4) were maintained in RPMI-1640 medium (Gibco-BRL; Invitrogen Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 mg/ml streptomycin. The cells were maintained as monolayers in 10-cm diameter cell culture dish at 37˚C in a humidified atmosphere of 5% CO_2 in air.

shRNA lentiviral transfection. CD9-targeted shRNA lentiviral plasmid (Mission; Sigma-Aldrich, target sequence: ccgggctg ttcggatttaacttcatctcgagatgaagttaaatccgaacagctttttg) and non-targeting control plasmid (pLKO.1-puro) were transfected with ViraPower™ Lentiviral packaging mix to cell lines using Lipofectamine 2000 (Invitrogen Life Technologies). The cells were transfected with the shRNA-expressing lentivirus, and stable cell lines were generated by selection with puromycin. Knockdown of CD9 was confirmed by FACS analysis with the FITC mouse anti-human CD9 antibody (BD Pharmingen).

In vitro migration assay. Migration assay was performed using a 24-well Boyden chamber with a non-coated 8-mm pore size filter in the insert chamber (BD BioCoat). Cells (CD9 shRNA- and control shRNA-transfected MSTO-211H) ($5x10^4$) were suspended in 0.5 ml RPMI-1640 media containing 0.1% FCS and seeded into the insert chamber. Cells were allowed to migrate for 48 h into the bottom chamber containing 1 ml of RPMI-1640 media containing 10% FCS in a humidified incubator at 37˚C in 5% CO_2. Migrated cells which had attached to the outside of the filter were visualized by staining with Diff-Quik (International Reagents Co.) and counted.

Patients and tissue specimens. One hundred and twelve cases of malignant pleural mesothelioma were retrieved from the archival pathology files of the Department of Pathology, Graduate School of Biomedical Sciences, Hiroshima University. Small biopsy specimens were not included in this study. The histological diagnosis of mesothelioma was previously carried out by three independent pathologists (V.J.A., Y.T., K.I.) based on WHO criteria (15) and were confirmed in all instances by clinical, histological and immunohistochemical findings. Epithelioid mesothelioma (EM) was further subdivided into two subtypes, i.e., 'differentiated' type (EM-D) and 'less-differentiated' type (EM-LD) based on the morphology of 'papillo-tubular structures' as an indicator of differentiation (16). Thus, EM-D were EMs showing a papillo-tubular pattern, micropapillary pattern and/or microcystic pattern and EM-LD were EMs showing solid nest, trabecular pattern, signet-ring cell-like appearance and/or single-cell infiltration pattern. The histological classification was carried out prior to this study. The clinical data of the patients were retrieved from the hospital records. This study was carried out in accordance with the Ethical Guidelines for Epidemiological Research enacted by the Japanese Government as tissue specimens were collected and carried out strictly to protect personal identity after approval by the Institutional Review Board at Hiroshima University.

Immunohistochemistry. Immunohistochemical stainings were performed on 3-μm paraffin sections using the monoclonal anti-CD9 antibody. Tissue sections were deparaffinized, hydrated and endogeneous peroxide was quenched using 0.3% hydrogen peroxidase for 30 min. Sections were incubated in a humidified chamber with mouse monoclonal anti-CD9 antibody (diluted 1:100, clone 72F6, NB110-41534; Novus Biologicals, Littleton, CO, USA) overnight at 4˚C. The reaction was visualized using the Histofine Simple Stain kit (Nichirei Biosciences, Tokyo, Japan) with diaminobenzidine as a chromogen and nuclear counterstaining with Mayer's hematoxylin. A similar immunohistochemical procedure was carried out with the omission of the primary antibody as a negative control. Endothelial cells in and around the tumor tissue were considered as the internal positive control for validation of the immunohistochemistry. The membranous staining of CD9 was scored as 0, no staining; 1^+, 1-10%; 2^+, 10-50%; and 3^+, >50% of tumor cells immunostained in the tissue sections. Immunohistochemical scoring was carried out by two pathologists (V.J.A., Y.T.) independently without knowledge of the clinicopathologic or disease outcome variables. Multiple sections from different paraffin blocks were analyzed for CD9 expression to confirm negative CD9 expression.

Statistical analysis. Fisher's exact test and Pearson Chi-square test were used for association analyses of the clinicopathological parameters with CD9 expression. Univariate analysis and multivariate Cox proportional hazards regression analysis for overall survival were performed with the regressors: CD9 expression, age, gender, clinical IMIG staging, histological type, differentiation, therapeutic regimen, extrapleural pneumonectomy and chemotherapy status. The overall survival curves of patients with follow-up data were estimated using the Kaplan-Meier method. Multivariate Cox proportional hazard ratio (HR) was separately calculated for all mesotheliomas and EMs for patients with clinical data for all parameters including age, histology (in analyzing all cases)/differentiation (in analyzing EM alone), IMIG staging and therapeutic regimen. Statistical analysis of the migration assay was performed by the two-tailed t-test. All of the statistical analyses were carried out using JMP 9.0 software. P-value <0.05 was considered to indicate a statistically significant result.

Results

Effect of shRNA knockdown of CD9 on cell migration. Immunohistochemical analysis of CD9 expression in the patient mesothelioma samples indicated that CD9 expression was a favorable prognostic factor. Therefore, we investigated the effects of CD9 on the cell migration of mesothelioma cell lines using shRNA-mediated CD9 knockdown. Knockdown of CD9 in the MSTO-211H and TUM1 cell lines was confirmed by FACS analysis, and the migration of cells was analyzed using Boyden chamber assay (Fig. 1). CD9 shRNA-transfected and control shRNA-transfected MSTO-211H and TUM1 cells were allowed to migrate toward medium containing 10% FCS as a chemoattractant. CD9 shRNA-transfected MSTO-211H and TUM1 cells showed increased migration in comparison to the control shRNA-transfected cells (Fig. 1).

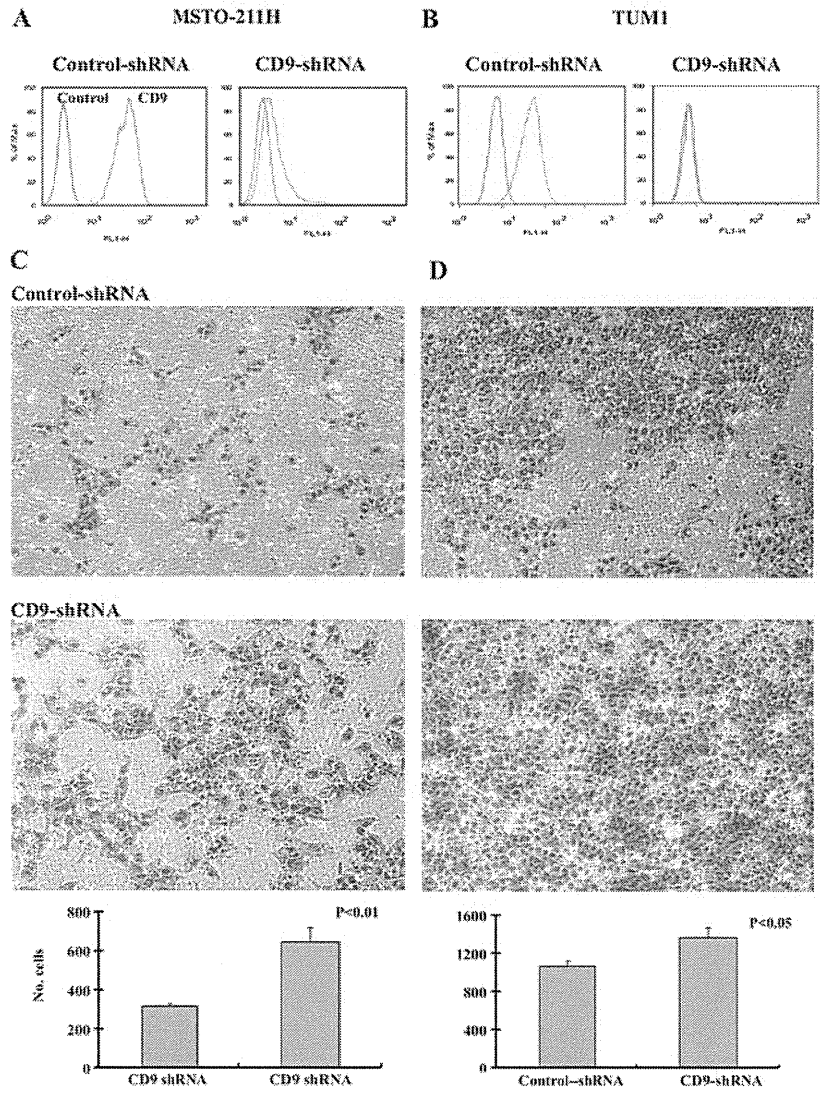

Figure 1. Flow cytometric analysis of CD9 expression in (A) MSTO-211H and (B) TUM1 cells transfected with control-shRNA and CD9-shRNA. Successful knockdown of CD9 was confirmed. Boyden chamber migration assay showed significantly increased cell migration of (C) MSTO-211H and (D) TUM1 cells transfected with CD9-shRNA (lower panels) in comparison to the control-shRNA (top panels).

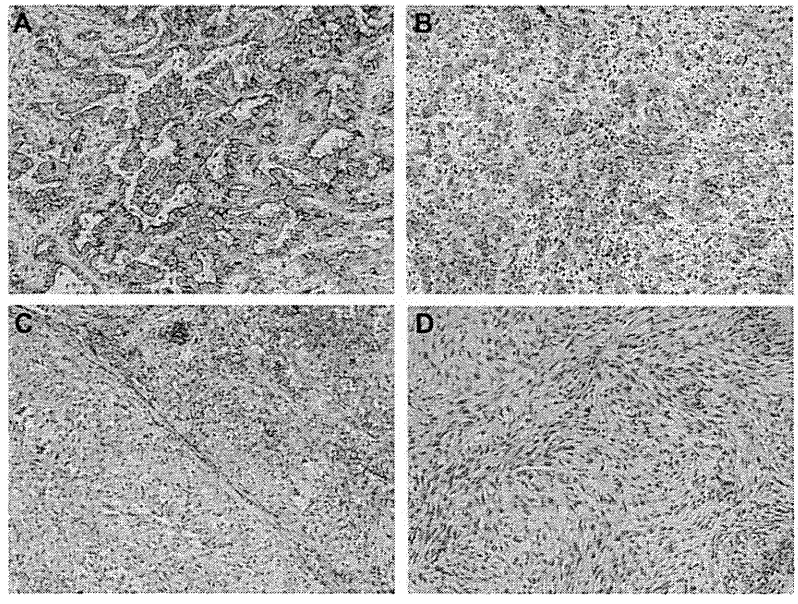

Figure 2. CD9 immunohistochemistry. (A) Epithelioid mesothelioma differentiated type showing diffuse CD9 expression. (B) Epithelioid mesothelioma less differentiated type showing decreased CD9 expression. (C) Biphasic mesothelioma showed CD9 expression in the epithelioid component while CD9 expression was not evident in the sarcomatoid component. (D) CD9 was not evident in the sarcomatoid component of sarcomatoid mesothelioma as well.