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Abbreviations: ADA: adenosine deaminase; APC: antigen-presenting cells; alloSCT: allogeneic stem cell transplantation; BMT: bone marrow transplantation; CBD: caveolin-binding domain; DPPIV: dipeptidyl peptidase IV; GLP-1: glucagon-like peptide 1; GVHD: graft-versu-host disease; IRAK-1: interleukin-1 receptor associated serine/threonine kinase 1; M6P/IGF-IIR: mannose 6-phosphate/insulin-like growth factor II receptor; NT-Fc: soluble Fc fusion proteins containing the N-terminal domain of caveolin-1; PMA: pokeweed mitogen; RA: rheumatoid arthritis; rsCD26: recombinant soluble CD26; TT: tetanus toxoid; mAb: monoclonal antibody; Tollip: Toll-interacting protein; TCR: T-cell receptor

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The role of CD26/dipeptidyl peptidase IV in cancer

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1. ABSTRACT

CD26/DPPIV is a multifunctional cell surface protein that is widely expressed in most cell types including T lymphocytes, on which it is a marker of activation. It is also present in serum and other body fluids in a truncated form (sCD26/DPPIV). It preferentially cleaves N-terminal dipeptides from polypeptides with proline or alanine in the penultimate position, and in doing so, regulates the activities of a number of cytokines and chemokines. Due in part to this ability to regulate the activity of biopeptides, it can act as a tumor suppressor or activator. It can associate with several proteins, among them fibroblast activating protein-alpha (FAP-alpha), plasminogen, adenosine deaminase (ADA), the tyrosine phosphatase CD45, and the chemokine receptor CXCR4. It can also bind to the extracellular matrix (ECM) and depending on the presence of other ligands, this process can either lead to increased or decreased invasive activity of the cells on which it is expressed. As a result of these characteristics, CD26/DPPIV plays an important role in tumor biology, and is useful as a marker for various cancers, with its levels either on the cell surface or in the serum being increased in some neoplasms and decreased in others. Our group has shown that CD26/DPPIV can be manipulated by such agents as CD26 cDNA-carrying plasmids, siRNA and monoclonal antibodies, resulting in both *in vitro* and *in vivo* inhibition of cell growth, enhanced sensitivity to selected chemotherapeutic agents, and enhanced survival of mouse xenograft models. These studies have demonstrated the utility of these tools as potential targeted therapies for specific cancers expressing CD26/DPPIV.

2. INTRODUCTION

CD26 (DPPIV) is a multifunctional membrane-bound glycoprotein present on the surface of most cell types. It is a type II cell surface protein, as the bulk of the protein including the carboxy terminus faces the extracellular space. As a dipeptidyl peptidase, it preferentially cleaves N-terminal dipeptides from polypeptides with proline or alanine in the penultimate position. This activity places it in the prolyl peptidase family and is responsible for its best known functions—chemokine regulation and glucose homeostasis (1). Its enzymatic activity, which is responsible for many but not all of its activities, resides in the carboxy terminal extracellular domain and is involved in the activation/inactivation of a number of chemokines and cytokines. CD26/DPPIV has 3 domains: an extracellular domain, a transmembrane region, and a short cytoplasmic tail of 6 amino acids. Enzymatically active CD26/DPPIV is a homodimer, each subunit containing an alpha/beta hydrolase domain and a beta-propeller domain. A large cavity formed by the alpha/beta hydrolase and the eight-bladed beta-propeller domain acts as the substrate binding site (2). CD26/DPPIV was initially considered to cleave only after a proline or alanine residue, but its substrates now include hydroxyproline, serine, glycine, valine, threonine, and leucine. The discrimination between proline and alanine in the penultimate position is much greater for the dipeptide chromogenic and fluorogenic substrates than for its natural substrates (3). In addition to its exopeptidase activity, there is some evidence that it also has endopeptidase activity, which could play a role in extracellular matrix degradation and hence invasion (4). However, it is possible that this activity is mediated through CD26/DPPIV association with FAP-alpha (5).

A soluble form of the protein (sCD26/DPPIV) is present in the serum and other body fluids, presumably as a result of shedding or secretion from different cell types. The soluble form lacks the transmembrane region and cytoplasmic residues, as it begins at amino acid 39 (6). This form is also a dimer and has been detected in seminal fluid as a larger oligomer (>900 kDa) (7).

3. PROTEINS ASSOCIATED WITH CD26/DPPIV

CD26/DPPIV also exhibits biological functions that are unrelated to its dipeptidase activity. With its beta-propeller domain containing binding sites for several proteins, in addition to forming heterodimers with FAP-alpha, CD26/DPPIV has also been reported to associate with plasminogen 2, ADA, CD45, CXCR4, and mannose 6-phosphate/insulin-like growth factor II receptor (mannose-6-P/IGFIIR). Furthermore, it can bind to the ECM proteins fibronectin and collagen.

3.1. FAP-alpha (Seprase)

FAP-alpha cDNA also codes for a type II integral membrane protein with a large extracellular domain, a transmembrane segment, and a short cytoplasmic tail with 48% amino acid sequence identity with CD26/DPPIV. Although the active form of CD26/DPPIV is a homodimer, expression of both proteins in COS-1 cells resulted in the formation of heterodimeric complexes (8). The genes for both proteins are located in the 2q23 region, suggesting that they may have arisen by gene duplication. However, their expression patterns are different, with CD26/DPPIV being constitutively expressed in most tissues (except in the case of T and B cells, where its expression is regulated by activation), and FAP-alpha expression being more restricted. FAP-alpha has been found to be localized at the invasion front during invasion into the extracellular matrix by human melanoma (9), breast carcinoma (10), and endothelial cells (5). Formation of the CD26/DPPIV-FAP-alpha complex at invadopodia of migrating fibroblasts was required for cell invasion on a collagenous matrix. In addition, antibodies to the gelatin-binding domain of CD26/DPPIV reduced cell migration and degradation of collagen (11). Interestingly, CD26/DPPIV transfectants have been shown to induce FAP-alpha (12, 13).

3.2. Plasminogen 2

Plasminogen 2 (Pg 2) binding to CD26/DPPIV was first demonstrated in rheumatoid arthritis synovial fibroblasts (14). Pg 2 bound to CD26/DPPIV residues 313-319 (15) and was dependent on the sialic acid content of the plasminogen isoform. Pg 2-gamma, Pg 2-delta, and Pg 2-epsilon bound to CD26/DPPIV, whereas Pg 2-alpha and Pg2-beta did not bind. However, in the prostate tumor cell line 1-LN, only Pg 2-epsilon induced expression and secretion of metalloproteinase 9 (MMP-9) (16). Hence, the ability of CD26/DPPIV to associate with plasminogen may be a factor in the invasiveness of certain cancers.

3.3. ADA

ADA catalyzes the deamination of adenosine and deoxyadenosine to inosine and 2'-deoxyinosine, respectively. ADA is located in both the cytosol and on the surface of lymphocytes, where it is associated with CD26/DPPIV (17) and dependent on CD26/DPP IV expression. The function of ADA located on the cell surface is to regulate extracellular adenosine and deoxyadenosine, which are toxic to lymphocytes (18). Adenosine, which accumulates in the extracellular fluid of solid tumors, caused down-regulation of CD26/DPPIV in HT29 colorectal carcinoma cells, which resulted in the depletion of ADA on the cell surface and consequently, a further increase in adenosine. Since the presence of excess adenosine suppresses the immune system, this process may facilitate tumor survival (19). Meanwhile, a different response was reported in endothelial cells. In this case, although hypoxia led to an increase in adenosine level, ADA and CD26/DPPIV mRNA and protein expression were likewise upregulated. The differential effect of adenosine on ADA and CD26/DPPIV in these distinct experimental conditions may be partially due to differences in adenosine levels and also tissue types (20).

The crystal structure of the ADA-CD26/DPPIV complex has revealed binding of one ADA molecule to each beta-propeller domain of CD26/DPPIV (21). The crystal structure also indicates that tetramerization of CD26/DPPIV is a key mechanism for the regulation of its interaction with other components (22). It has been suggested that binding of ADA could regulate

CD26/DPPIV-mediated adhesion between cells by inhibiting tetramer formation between dimers. This is consistent with an earlier study which showed that addition of exogenous ADA inhibited adhesion between lymphocytes and epithelial cells promoted by CD26/DPPIV (23).

3.4. CD45

CD45 is a membrane tyrosine phosphatase that regulates Src-family kinases. It is highly expressed in hematopoietic cells where it chiefly regulates Lck activity. CD45 can regulate Lck activity (important for TCR signaling events) by dephosphorylation of Tyr 505, the negative regulatory site, or down-regulate Lck by dephosphorylation of Tyr 394 in the kinase domain (24). In lymphocytes, CD26/DPPIV and CD45 have been shown to be associated through the binding of CD26/DPPIV to the cytoplasmic domain of CD45 (25, 26). Furthermore, cross-linking of CD26 by a CD26-specific antibody leads to increased phosphorylation of several cell signaling proteins, including $p56^{lck}$, $p59^{fyn}$, ZAP70, and MAP kinase, most likely due to its association with CD45 (27). CD45 has also been implicated in the negative regulation of CD44-mediated cell spreading (28).

3.5. CXCR4

The consequences of the association between the chemokine receptor CXCR4 and CD26/DPPIV are less well understood. Stromal cell-derived factor-1-alpha (SDF-1-alpha)/CXCL12 and its receptor, CXCR4, have recently been shown to play a critical role in tumorigenesis in addition to their well-recognized role in mediating migration and activation of leukocytes during immune and inflammatory responses (29). CXCR4 is upregulated in hypoxic tissues (30) and is the main receptor for SDF-1-alpha, a chemokine which attracts cells expressing CXCR4 and which is cleaved by CD26/DPPIV. Binding of SDF-1-alpha triggers internalization of both CXCR4 and CD26/DPPIV in the T-cell line, Jurkat J32, the B-cell line, SKW6.4, and peripheral blood lymphocytes (31), suggesting that these two proteins may function together.

3.6. Mannose-6-P/IGFIIR

CD26/DPPIV binds to mannose-6-P/IGFIIR via mannose-6-phosphate residues in the carbohydrate moiety of CD26/DPPIV (32). This interaction is critical for CD26/DPPIV-mediated T cell activation and migration (32, 33). Endothelial cells expressing mannose-6-phosphate/IGFIIR on their surface bind to mannose-6-P on sCD26, indicating that sCD26/DPPIV is involved in T-cell migration via its interaction with mannose-6-P/IGFIIR. Moreover, enhanced migration was dependent on DPPIV enzyme activity. Although CD26/DPPIV has no known motif for endocytosis, upon T cell activation, mannose-6-phosphorylation increases, leading to increased binding to the mannose-6-P/IGFIIR and resulting in CD26/DPPIV internalization. Internalization of CD26/DPPIV can also occur following SDF-1-alpha binding to CXCR4, which causes cointernalization of CXCR4 and CD26/DPPIV (31). Of note is that internalization of both CXCR4 and CD26/DPPIV require phosphorylation; for CD26/DPPIV phosphorylation of sugar residues (32), and for CXCR4, phosphorylation of serine residues (34).

3.7. Collagen and Fibronectin

Both collagen and fibronectin are components of the extracellular matrix. The interaction between collagen and CD26/DPPIV is mediated by residues in the cysteine-rich region of CD26/DPPIV and not the catalytic domain (35). CD26/DPPIV also binds to fibronectin (36-38). A detailed study of the binding of CD26/DPPIV to fibronectin was carried out using fibronectin fragments, which found this process to be mediated by the consensus motif T (I/L)TGLX (P/R)G (T/V)X (37). CD26/DPPIV binding to fibronectin is important for adhesion to specific cell types. It is thought that cancer cells initially arrest in the microvasculature of the first organ they encounter with only a few cells forming metastases at a particular secondary site. Breast carcinomas most frequently metastasized to the lungs, for example, and were shown to bind to lung endothelia expressing CD26/DPPIV mediated by the fibronectin assembled on their surface (36).

4. DIFFERENCES BETWEEN CD26/DPPIV AND OTHER FAMILY MEMBERS

4.1. FAP-alpha (Seprase)

FAP-alpha was first identified in the malignant melanoma cell line LOX on the basis of its presence in invasive cell lines. It was also found in membrane vesicles obtained from conditioned media from this cell line. However, it was not detected in a control melanoma cell line or in 32 other tumor cell lines that were unable to degrade extracellular gelatin (39). Similar to CD26/DPPIV, FAP-alpha is a type II membrane protein and a member of the prolyl peptidase family, but unlike CD26/DPPIV, it is inhibited by cysteine protease inhibitors, such as N-ethylmaleimide (39). Also, dipeptides that are CD26/DPPIV substrates are cleaved with a substantially lower catalytic efficiency (100-fold) by FAP-alpha, due to the presence of different amino acids in its active site. In contrast to CD26/DPPIV, FAP-alpha has both gelatinase and collagenase activities (40). In addition, it is generally not expressed in normal tissue, but is present in epithelial cancers and reactive stromal fibroblasts of bladder, breast, colorectal, lung and ovarian carcinomas (41).

Although FAP-alpha is not expressed in normal adult tissues, it can be induced in fibroblasts in response to wounding and also in the reactive stroma of epithelial cancers and some sarcomas (41). Its expression in some malignant cells of epithelial origin, e.g. invasive ductal carcinoma cells from breast cancers, gastric carcinoma, and melanoma, has been demonstrated in several recent studies (10, 39, 42).

Human embryonic kidney cells (HEK293) transfected with murine FAP-alpha and injected into mice formed tumors that grew more rapidly than tumors from control vector-transfectants. Rapid growth depended on protease activity, and was negated by antibodies that inhibited dipeptide cleavage (43). Human breast cancer

cells transfected with FAP-alpha cDNA also formed tumors that grew more rapidly than tumors from transfectants not expressing FAP-alpha (44). In addition, the fast growing tumors exhibited a higher microvessel density. However, there was no difference in *in vitro* proliferation between parental and FAP-alpha-expressing cells. Data from this particular study would support the conclusion that FAP-alpha drives angiogenesis. Additional support for this idea was provided by another study showing that FAP-alpha mRNA upregulation by endothelial cells was involved in capillary morphogenesis (45). Interestingly, non-small-cell lung carcinoma (NSCLC) cells transfected with either a mutant lacking DPPIV activity (S630A) or wild-type CD26/DPPIV induced expression of FAP-alpha (13).

4.2. DPP8 and DPP9

It is likely that DPP8 and DPP9 contribute to biological functions previously attributed to CD26/DPPIV. Both DPP8 and DPP9 have been expressed in baculovirus and shown to function as dimers with similar kinetic and substrate profiles (46). When transfected into 293T cells, a cell line that does not express FAPα and expresses CD26/DPPIV intracellularly at low levels, both DPP8 and DPP9 localized to the cytoplasm. Furthermore, cell migration and monolayer wound healing were impaired by overexpression of either DPP8 or DPP9 (47). Using selective inhibitors, it has been shown recently that DPPIV activity attributable to DPP8/9 is present in human peripheral blood mononuclear cells (PBMC). Most of the DPP8/9 activity was localized to the cytosol, whereas CD26/DPPIV activity was concentrated in the membrane (48).

4.3. DPP10 (DPL2)

Although this protein shares homology with CD26/DPPIV (32%), the active site serine residue is replaced by glycine, resulting in loss of enzyme activity. In contrast to CD26/DPPIV, it is not widely expressed but is present chiefly in brain and pancreas (49, 50).

Based on experiments with CD26/DPPIV enzyme inhibitors, it has become clear that CD26/DPPIV-related family members are targets of drugs initially thought to be specific for CD26/DPPIV. For example, the dipeptidyl peptidase inhibitor, val-boro-pro triggered tumor regression and rejection in WEHI 164 fibrosarcoma and EL4 and A20/2J lymphoma models. Furthermore, treatment with this inhibitor induced upregulation of cytokine and chemokine expression in the tumor and draining lymph nodes. Antitumor activity and stimulation of cytokine and chemokine production was unchanged in $CD26^{-/-}$ mice (51). Therefore, it is currently thought that this DPPIV inhibitor targets FAP-alpha in the tumor stroma as well as cytoplasmic DPP8 and DPP9, inducing a cytokine-mediated immunological response.

5. CD26/DPPIV EXPRESSION IN HUMAN CANCERS

The exact role CD26/DPPIV plays in various 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. Furthermore, given the plethora of its 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.

5.1. Cancers associated with high CD26/DPPIV expression

5.1.1. Mesothelioma

CD26/DPPIV was shown to have the highest activity among cell surface aminopeptidases in human mesothelial cells (52). Since mesothelial cells are in constant contact with bodily fluids such as ascites, the influence of ascites on DPPIV activity was measured. Mesothelial cells cultured in the presence of malignant ascites from ovarian carcinoma patients exhibited an increase in DPPIV activity of up to 200% over the control, whereas no significant increase was observed for benign ascites. Fractionation of the ascites revealed that the activity was present in the fraction containing small peptides (<3 kD) and was responsible for elevating both CD26/DPPIV mRNA and protein expression on the cell surface (52). More recent studies confirm that CD26/DPPIV is expressed at a high level on the surface of malignant mesothelioma cells, but not on cells derived from a benign mesothelioma (53). Malignant mesothelioma is an aggressive cancer involving the mesothelioma cells lining the pleura and is resistant to conventional treatments. Depletion of CD26/DPPIV by siRNA resulted in loss of binding to ECM proteins, fibronectin and collagen. Similarly, our recent work showed that incubation with the murine monoclonal antibody 14D10 or a humanized antibody against CD26/DPPIV caused loss of binding to ECM proteins and upregulation of $p27^{kip1}$. Antibody treatment of mice inoculated with human malignant mesothelioma cells inhibited tumor growth and enhanced survival when either 14D10 or the humanized CD26 antibody was injected, whereas the isotype matched control had no effect. In addition, both CD26/DPPIV-specific antibodies reduced formation of metastases (53).

5.1.2. Renal

CD26 has also been shown to be expressed on renal carcinoma cells (54, 55), including the cell lines Caki-1, Caki-2, ACHN, and VMRC-RCW (56). We recently treated Caki-2 cells, which strongly express CD26/DPPIV, with the murine monoclonal anti-CD26 antibody 14D10. Following treatment, cells arrested in G1/S, accompanied by an induction of $p27^{kip1}$, down-regulation of cyclin-dependent kinase 2, and dephosphorylation of retinoblastoma protein. When mice inoculated with human renal carcinoma cells were injected with the CD26-specific antibody, tumor growth was inhibited and survival was significantly enhanced (56).

5.2 . Cancers associated with variable CD26/DPPIV expression

5.2.1. Colon cancer

CD26/DPPIV level was found to be correlated with a differentiated phenotype in both HT-29 and Caco-2 colon cancer cells (57). Neither protein stability nor glycosylation was affected by the state of differentiation. Instead, expression appeared to be controlled at the transcriptional level, since CD26/DPPIV mRNA level was low in undifferentiated cells, but increased as differentiation progressed. Although CD26/DPPIV is not expressed in the adult colon, it has been shown in some cases to be re-expressed in some colon cancers and cell lines.

CD26/DPPIV was down-regulated by adenosine in HT-29 colorectal carcinoma cells, a process mediated by an increase in tyrosine phosphatase activity leading to decreased tyrosine phosphorylation of MAP kinase ERK (1/2) (58). Another study showed that while CD26/DPPIV expression was lower in the colon carcinoma cell lines tested, FAP-alpha was expressed at higher levels in cancer cells and adjacent stromal cells compared to normal colorectal tissue. In addition, a correlation was found between FAP-alpha expression and lymph node metastasis, with high FAP-alpha expression in colorectal cancer tissue being associated with lymph node metastasis (59, 60).

Conflicting reports have been published regarding the presence of soluble CD26/DPPIV molecules in the serum of patients with colorectal carcinoma. One study indicated that serum levels in healthy donors were significantly higher than those in colorectal carcinoma cancer patients (61). However, in another report, a higher level of soluble CD26/DPPIV was detected in patients diagnosed with colorectal cancer, being highest in those with metastatic disease (62). While the reason for the observed difference is unclear, one possibility may be due to the detection methods used-an ELISA assay in the former, and an assay for enzyme activity in the latter.

Meanwhile, tetraspanins are integral membrane proteins that play a role in organizing multimolecular complexes in the plasma membrane. Several studies have demonstrated a link between the expression of these proteins and metastasis. In a recent study utilizing a colon cancer model consisting of cell lines derived from the primary tumor and two metastases, CD26/DPPIV and the tetraspanin Co-029 (identified by mass spectrometry) were present only on the metastatic cancer cells (63).

5.2.2. Glioma

In a recent study using glioma cell lines, no simple correlation could be demonstrated between CD26/DPPIV expression and the degree of malignancy. The lack of an unambiguous result was due to the contribution of DPPIV-like enzymatic activity contributed by dipeptidyl peptidase IV activity and/or its structural homologs (DASH) (64). In an earlier report by the same group of investigators, a positive correlation had been made between the degree of transformation and DPPIV activity, but at the time the contribution of other DPPIV-like proteins was not fully appreciated (65).

5.2.3. Hematological malignancies

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. CD26/CPPIV could also be induced in normal B cells following treatment with interleukin-4, with RT-PCR analysis indicating that expression was regulated at the level of transcription (66). In contrast, the expression of CD26/DPPIV was decreased in the PBMC of patients with adult T cell leukemia/lymphoma (ATLL) compared with cells from normal donors. Again, expression was regulated at the level of transcription (67). Down-regulation appears to result from methylation of CpG islands in the promoter region (68).

CD26/DPPIV has also been shown to be a marker for aggressive T-large granular lymphocyte lymphoproliferative disorder (T-LGL LPD). 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. Furthermore, CD26-related signaling may be aberrant in T-LGL as compared to T-lymphocytes from normal donors (69). Disease aggressiveness is also correlated with CD26/DPPIV expression in other subsets of T-cell malignancies including T-lymphoblastic lymphoma/T-acute lymphoblastic leukemia (LBL/ALL), as those with CD26-positive T-LBLALL had a worse clinical outcome compared to patients with CD26- negative tumors (70, 71).

Two CD26/DPPIV inhibitors were used to elucidate the function of CD26/DPPIV in clones of the human histiocytic lymphoma cell line U937 expressing different levels of CD26/DPPIV. The presence of these inhibitors suppressed DNA synthesis and cytokine production in high expressors while having no effect in low expressors, and these findings support the notion that CD26/DPPIV plays a role in the proliferation and cytokine production of transformed cells (72). Meanwhile, we showed that treatment with anti-CD26 monoclonal antibody inhibited adhesion of the human CD30+ anaplastic large cell T-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 (38). We also showed that treatment with anti-CD26 monoclonal antibody inhibited the growth of T -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 (73). Likewise, we demonstrated that anti-CD26 monoclonal antibody treatment of the CD26-positive T-lymphoma line Karpas 299 resulted in *in vitro* and *in vivo* antitumor activity, with associated enhanced survival of SCID mice inoculated with Karpas 299 cells (74).

On the other hand, CD26/DPPIV was not detectable on tumor samples from most patients with the T-cell lymphoma subtype mycosis fungoides/Sezary syndrome, with low expression in the remaining patients in one published study (75). These findings have been corroborated by other investigators (76, 77). Therefore, the absence of CD26/DPPIV expression can be used in the clinical setting as a marker for the diagnosis of mycosis fungoides/Sezary syndrome, as suggested by our work (75). Meanwhile, a recent study suggested that the skin-homing characteristic of Sezary syndrome tumor cells is mediated by the interaction between the chemokine receptor CXCR4 and its ligand SDF-1-alpha, and is influenced by the fact that Sezary syndrome cells do not express CD26/DPPIV. The presence of exogenously added soluble CD26/DPPIV led to cleavage of SDF-1-alpha, interference with SDF-1alpha-CXCR4 interaction, and decreased SDF-1-alpha-

mediated migration of Sezary syndrome cells. Conversely, inhibition of DPPIV enzyme activity induced SDF-1-alpha-mediated cell migration. Therefore, the CD26/DPPIV-regulated interaction of SDF-1-alpha-CXCR4 could play a key role in the skin-homing ability of Sezary syndrome cells and be responsible for the cutaneous manifestations typically associated with this disease in the clinical setting (78).

Our recent work with B and T-tumor cell lines also demonstrated an association between CD26/DPPIV and the key intracellular protein topoisomerase II alpha, which is a target for such widely used topoisomerase II inhibitors as doxorubicin and etoposide. Overexpression of CD26/DPPIV by the use of stable transfectants in the CD26-negative T-leukemia line Jurkat and B-lymphoma line Jiyoye resulted in increased p38 phosphorylation and enhanced level of topoisomerase II alpha, associated with increased sensitivity to the topoisomerase II inhibitors (79-82). Conversely, depletion of CD26/DPPIV expression through small interfering RNA transfection of the CD26-positive T-cell lymphoma line Karpas 299 resulted in dephosphorylation of p38 and decreased topoisomerase II alpha level, leading to decreased sensitivity to doxorubicin and etoposide (38). Since the topoisomerase II inhibitors doxorubicin and etoposide are chemotherapeutic agents that are widely used in hematologic malignancies, these findings have potentially important implications in the clinical setting. Also importantly, siRNA-mediated down-regulation of CD26 resulted in decreased tumorigenicity of Karpas 299 cells in a SCID mouse xenograft model, implying a direct role for CD26 in tumor growth and development (38). These findings further support a therapeutic approach involving targeted therapy against CD26 for selected human cancers.

Certain hematological malignancies respond well to ADA inhibitors, suggesting that the interaction between CD26/DPPIV and ADA might play a role in tumor progression. Since pentostatin (2'-deoxycoformycin) is a potent inhibitor of ADA and CD26/DPPIV expression is integral to ADA cell-surface expression and function, the effect of pentostatin was tested on human leukemia/lymphoma T cell lines expressing different levels of CD26/DPPIV (83). Since *in vitro* exposure to pentostatin alone was usually insufficient to slow growth and induce apoptosis, adenosine or deoxyadenosine was used in conjunction with pentostatin. These studies unexpectedly showed that the expression of CD26 was inversely correlated with the ability of pentostatin to inhibit tumor cell growth and induce apoptosis. Meanwhile, our work showed that treatment of T-lymphoma patients with pentostatin resulted in a specific reduction in the level of circulating CD26-positive T-lymphocytes, potentially associated with immunosuppression (84).

5.2.4. Hepatocellular carcinoma

Cytochemical analysis showed that all hepatocellular carcinomas displayed an altered distribution of CD26/DPPIV activity, with the appearance of three representative patterns: distorted canaliculi with abnormally high activity; loss of activity in canaliculi, with activity restricted to isolated spots; and pseudoacinar structures of hepatocytes with basolateral and apical activity. For most pathological non-neoplastic liver disease, distribution did not significantly differ from that found in normal liver, and was localized in the bile canalicular plasma membrane (85).

In rat liver tissue, CD26/DPPIV enzyme activity in the plasma membranes of two different hepatomas was only 3% of the activity measured in normal livers. In contrast, CD26/DPPIV activity in the serum of rats with hepatomas was on average 150% of the normal value. Immunofluorescence staining with a CD26/DPPIV antibody revealed loss of surface expression, suggesting that CD26/DPPIV was shed into the serum (86).

5.2.5. Lung cancer

CD26 expression in lung cancer appears to be dependent on the specific histologic subtype. When the expression of CD26/DPPIV was compared at the mRNA and protein levels in non-small-cell lung cancer cell lines and normal bronchial epithelial cells, CD26/DPPIV was detected in normal epithelial cells, but was reduced or not detectable in NSCLC cell lines. Downregulation occurred at the RNA level (13). Lung adenocarcinoma appears to be the exception, as CD26/DPPIV is expressed only in this subtype, suggesting that it might be useful as a marker to distinguish it from other types of lung cancers (87). Interestingly, multiple molecular forms of CD26/DPPIV are observed in normal and cancerous lung tissues. CD26/DPPIV from lung cancer tissue consists of more basic molecular forms than that from normal lung tissue, suggesting that the molecular properties of CD26 in the two types of lung tissues are different (88).

5.2.6. Prostate cancer

Studies evaluating the expression of CD26/DPPIV in prostate cancer tissue have yielded mixed results. In one study, CD26/DPPIV activity was found to be twice as high in prostate cancer tissue compared to benign prostatic hyperplasia tissue, as determined by biochemical and quantitative histochemical methods (89). Measurement of DPPIV activity in secretions and different tissue zones also revealed higher activities in patients with cancer (90). In contrast, a different group has shown that loss of CD26/DPPIV was correlated with an increase in basic fibroblast growth factor (bFGF) in metastatic prostate cancer cells. Re-expression of CD26/DPPIV reversed the expression of bFGF and downstream effectors of the bFGF pathway, MAP kinase (ERK1/2) and urinary plasminogen activator (u-PA) (91). The opposite trend was observed using a different cell type, 1-LN. Plasminogen 2 isoforms (Pg 2-gamma, Pg 2-delta, and Pg 2-epsilon) have been shown to bind to CD26/DPPIV via their sialic acid residues, leading to a $[Ca^{2+}]_i$ response (15). In 1-LN cells, although all three isoforms were capable of binding to CD26/DPPIV, only Pg 2-epsilon induced expression and secretion of MMP-9 which led to increased invasion (16). Incubation with monoclonal antibodies to CD26/DPPIV, MMP-9, or u-PA blocked Pg 2-mediated invasion.

5.2.7. Thyroid cancer

CD26/DPPIV was expressed in nearly all cases of thyroid follicular and papillary carcinoma, whereas a lower

percentage of follicular adenoma cases stained positive for CD26/DPPIV, suggesting its potential usefulness as a marker for distinguishing thyroid cancer from benign tumors (92, 93). In addition, one study suggested that the co-expression of CD26/DPPIV and galectin-3 proteins and mRNAs may help in the diagnosis of differentiated thyroid carcinoma as compared to normal thyroid tissues or benign thyroid lesions (94). In an attempt to detect proteins that might contribute to the aggressive behavior of anaplastic thyroid carcinoma, five cases were selected because both undifferentiated and differentiated areas were present. Decreased levels of CD26/DPPIV were detected in undifferentiated areas compared with the differentiated areas, suggesting that it may play a role in regulating tumor aggressiveness and serve as a marker of disease prognosis (95).

5.2.8. Ovarian cancer

Five ovarian cell lines, HRA, SKOV3, TAOV, NOS4, and NOS2 were compared with respect to their CD26/DPPIV expression and invasive potential. HRA and SKOV3 expressed a low level of CD26/DPPIV, but exhibited the highest invasive potential. In contrast, TAOV, NOS4, and NOS2 expressed a high level of CD26/DPPIV, but exhibited low invasive potential (96). Transfection of the SKOV3 cell line with CD26/DPPIV resulted in increased adhesion, however, migration and invasion were substantially reduced (97). In addition, nude mice inoculated with CD26/DPPIV-transfected SKOV3 cells lived approximately twice as long as those receiving the parental or vector-transfected cells. In a separate study, SKOV3 cells were transfected with CD26/DPPIV cDNA, and binding to various substrates was measured. The CD26/DPPIV transfectants bound both collagen and fibronectin-coated plates to a greater extent than the parental cell line or transfectants expressing vector only. Inhibition of DPPIV activity had no effect on adhesion. For CD26/DPPIV transfectants, adhesion rates to mesothelial cells were twice that of parental cells and cells transfected with empty vector (98). Further studies with CD26/DPPIV transfectants indicated that expression levels of MMP-2 and MT1-MMP were reduced, while tissue inhibitors of matrix metalloproteinases were enhanced (96).

5.3 Cancers associated with low CD26/CPPIV expression

5.3.1. Breast cancer

The role of CD26/DPPIV in breast cancer is an area for future research. CD26/DPPIV expressed on the surface of rat lung capillary endothelia was shown to be a receptor for rat breast cancer cells that display fibronectin on their surface. CD26/DPPIV enzyme activity was not involved in binding. Furthermore, the extent of binding to CD26/DPPIV-expressing lung cells was shown to be proportional to the amount of fibronectin on the breast cancer cells (36). In addition, peptides containing the fibronectin CD26-binding domain blocked the CD26/DPPIV-fibronectin interaction and decreased pulmonary metastasis of the breast cancer cells (37). These findings are consistent with an earlier study on the capacity of CD26/DPPIV to act as an adhesion molecule in which outside-out endothelial cell membrane vesicles were used as a model system. In this study, a monoclonal antibody was generated that prevented adhesion of lung-derived endothelial membrane vesicles to lung-metastatic breast and prostate carcinoma cells. This antibody was found to be specific for CD26/DPPIV (99). In studies with F344 rat substrains, cells expressing lower levels of CD26/DPPIV exhibited lower *in vivo* adhesion and fewer colonies in lung tumors, following i.v. inoculation of rat syngeneic mammary adenocarcinoma cells, MADB106. It is not clear whether CD26/DPPIV in MADB106 cells played a role in adhesion or metastasis since these cells did not express CD26/DPPIV *in vitro* but acquired expression following injection into rats (100).

5.3.2. Endometrial cancer

CD26/DPPIV was found to be expressed on normal endometrial glandular cells, but its expression on endometrial adenocarcinoma was down-regulated with increasing grade of neoplasm. Therefore, downregulation of CD26/DPPIV expression was correlated with neoplastic transformation and tumor progression (101).

5.3.3. Melanoma

While CD26/DPPIV is present at high levels on normal melanocytes, the process of malignant transformation results in the loss of expression, which occurs at the stage when melanocytes become independent of exogenous growth factors (12, 102). Interestingly, re-expression of CD26/DPPIV rescued expression of FAP-alpha (12). Since these two proteins form heterodimers, these findings suggest that expression of either one regulates the localization of the other. When matrigel invasion assays were used in a study involving two melanoma cell lines, LOX and C8161, parental cells and cells transfected with the empty vectors were found to be highly invasive. However, the invasiveness of cells transfected with CD26/DPPIV was reduced by more than 75%. Decreased metastatic potential did not appear to require either the 6 amino acid cytoplasmic tail or DPPIV activity (103). FAP-alpha expression has been shown to correlate with the invasive phenotype of human melanoma and carcinoma cells (104). LOX cells with higher levels of FAP-alpha exhibited a more invasive phenotype than those with lower levels (9).

5.3.4. Oral cancer

CD26/DPPIV activity in serum and expression on peripheral blood T lymphocytes are decreased in patients with oral cancers compared to normal controls (105, 106). To better understand the biochemical mechanism involved in CD26/DPPIV down-regulation, the effect of cytokines produced by the squamous cell carcinoma cell line KB was studied in peripheral blood T cells. The factor present in KB culture media which was responsible for CD26/DPPIV down-regulation in T cells, and decreased activity in serum, was identified as tumor growth factor beta 1 (TGF-beta 1) (107).

6. SUMMARY AND PERSPECTIVE

It is likely that the pleiotropic effects of CD26/DPPIV account for its varied roles in different

cancers. Among its key attributes are its ability to associate with other key molecules and its cleavage of biological factors to regulate their functions. For example, it can bind to plasminogen 2-epsilon, triggering an intracellular [Ca^{2+}] flux which leads to secretion/activation of MMP-9 in 1-LN cells (16). It can also form heterodimers with FAP-alpha, colocalizing at pseudopodia and causing secretion/activation of MMPs in migratory fibroblasts and endothelial cells (5, 11). Both of these activities suggest that CD26/DPPIV is involved in tumor invasiveness. On the other hand, its ability to bind fibronectin and collagen not only predicts a potential role in invasion, but also perhaps an inability to migrate due to tight cell-cell adhesions mediated by CD26/DPPIV, as in the case for melanoma cells (103). Cleavage of cytokines and chemokines by CD26/DPPIV also enables it to act as either a tumor suppressor or activator. For example, SDF-1-alpha is one of the best CD26/DPPIV substrates *in vitro*, but whether it contributes significantly to the metabolism of SDF-1-alpha *in vivo* needs to be further studied. While many bioactive peptides are qualified to be CD26/DPPIV substrates, substrate recognition and cleavage efficiency are probably regulated at least partly by the proteins associated with CD26/DPPIV and the tumor-specific microenvironment, which can modulate substrate accessibility to the enzyme active site. The local concentration of the putative substrate is also important for its interaction with CD26/DPPIV. Therefore, the specific biological functions of CD26/DPPIV are likely to vary depending on its location, tumor cell type, oligomeric state, and the concentration of ligands and cofactors. In addition, multiple isoforms exist for both soluble and membrane-associated CD26/DPPIV, factors which add another layer of complexity to the role of this multifaceted molecule in tumor biology. Meanwhile, its various functions in tumor development would indicate that CD26 may therefore be an appropriate novel target for cancer therapy. Indeed, our studies, as well as work done by others, suggest that targeting CD26/DPPIV with specific agents may be an effective therapeutic approach for selected cancers, which would be logical in view of the key role CD26/DPPIV plays in cancer biology.

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Abbreviations: DPPIV: dipeptidyl peptidase IV; FAP-alpha: fibroblast activating protein-alpha; ADA: adenosine deaminase; ECM: extracellular matrix; siRNA: small interfering RNA; Mannose-6-P/IGFIIR: mannose-6-phosphate/insulin-like growth factor II receptor; Pg 2: plasminogen 2; MMP-9: metalloproteinase 9; SDF-1-alpha: stromal cell-derived factor-1-alpha/CXCL12; HEK293: human embryonic kidney cells 293; NSCLC: non-small-cell lung carcinoma cells; DPP8: dipeptidyl peptidase 8; DPP9: dipeptidyl peptidase 9; DPP10: dipeptidyl peptidase 10; MAPK: mitogen-activated protein kinase; ERK: extracellular signal-regulated kinase; bFGF: basic fibroblast growth factor; u-PA: urinary plasminogen activator

Key Words: CD26, dipeptidyl peptidase IV, DPPIV, DPP4, FAP-alpha, cancer, Hematologic Malignancies, Targeted Therapy, Review

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Stem cell properties and the side population cells as a target for interferon-α in adult T-cell leukemia/lymphoma

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Abstract

The cancer stem cell theory suggests that chemoresistance and recurrence of tumors are often due to the similarity of stem cell properties between normal and cancer cells. Adult T-cell leukemia/lymphoma (ATLL) has poor prognosis, suggesting that ATLL cells possess common stem cell properties. We analyzed side population (SP), a characteristic stem cell phenotype, and CD markers in ATLL cell lines. We found that several lines contained SP with expressions of some hematopoietic stem cell markers. On the other hand, treatment with interferon (IFN)-α is sometimes effective in ATLL, particularly combined with other drugs. We examined its effect on ATLL cells and found that IFN-α significantly reduced the SP proportion. Moreover, CD25-positive cells and phosphorylation of STAT1/5 and ERK were upregulated during this process. These data suggest that their stem cell properties render ATLL cells therapy-resistant, and IFN-α exerts its clinical effect through a reduction of the SP cell population.

Keywords: Adult T-cell leukemia/lymphoma; Cancer stem cells; Side population; Interferon-α

Adult T-cell leukemia/lymphoma (ATLL) is one of the peripheral T-cell malignancies caused by the T-cell leukemia/lymphoma virus type-1 (HTLV-1). It is characterized by the clonal integration of the HTLV-I provirus in tumor cells. Recent studies in stem cell biology demonstrated that some cancers contain stem-like cells (cancer stem cells, CSC) as a small subpopulation within the tumor tissues. Among human hematologic malignancies, CSC population has already been identified in acute and chronic myeloid leukemia (AML, CML) [1,2].

Flow cytometric analysis of cell surface antigens serves as the main tool to identify the CSC, although Hoechst 33342 dye staining and flow cytometric analysis have recently been used to identify both cancer and tissue stem cells. These cells are termed side population (SP) and stem cells of various tissues, including hematopoietic stem cells (HSC) and cancers, are often enriched in the SP fraction [3]. CSC frequently possess multidrug resistant 1 (MDR1) or ATP-binding cassette (ABC) transporter activities and exhibit chemoresistance [4] and SP phenotype [5]. For example, CSC were identified in the SP fractions of C6 glioma [6] and hepatocellular carcinoma cell lines [7]. Moreover, a relationship between AML and SP cells has been demonstrated [8,9].

Stem cell properties, so-called "stemness", are defined to be a self-renewal capacity and multipotency. For brain tumors, although conflicting results have been recently reported [10], it was previously demonstrated that SP cells of the C6 glioma cell line possess both capacities [6]. C6 SP cells, but not non-SP cells, can generate both SP and non-

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SP cells. Moreover, only SP cells can produce both neurons and glial cells. These results suggest that C6 contains a minor subpopulation of CSC that is enriched in the SP.

For hematologic malignancies such as T- and B-acute lymphoblastic leukemia (T-, B-ALL), and ATLL, the significance of CSC is still unclear since the development of lymphoid cells requires more complex processes. While recent works have characterized candidates for B- and T-ALL progenitor cells as being cells that express CD34 and not lymphoid precursor markers [11,12], investigators have also found that small subpopulations lacking CD138 expression had greater clonogenic potential in multiple myeloma [13].

Meanwhile, the fact that ATLL is caused by infection of mature $CD4^+$ cells by HTLV-I suggests that ATLL cells are not directly derived from HSC. However, ATLL patients often have tumor cells displaying diverse appearances, implying that ATLL cells have differentiation-like potential. Furthermore, ATLL patients have extremely poor prognosis with frequent chemoresistance and disease recurrences, suggesting stem cell properties for ATLL cells.

On the other hand, treatment of ATLL resistant to conventional therapies with the cytokine interferon (IFN)-α in combination with other agents has demonstrated effectiveness. A combination of IFN-α and zidovudine (AZT) induces a high complete remission rate and prolonged survival [14]. In vitro assays also demonstrated that arsenic trioxide synergizes with IFN-α to induce apoptosis in HTLV-1 infected cells [15]. Although these mechanisms have not yet been well characterized, it is speculated that IFN-α affects the stem cell properties, resulting in renewed drug sensitivity.

In the present study, we first searched for potential stem cell properties in ATLL cells by conducting SP and extensive surface antigen analysis in cell lines. We found that some ATLL cell lines contained SP cells and that they expressed several HSC-related markers, including CD48 [16] and CD90 (Thy-1) [17]. Additional analyses revealed that SP cells were reduced by treatment with IFN-α. During this process, an increase in the level of cells expressing the activated T-cell marker CD25 antigen and enhanced phosphorylation of STAT1/5 and ERK were observed, implying that SP phenotype is correlated with JAK/STAT and MAP kinase pathways. These results hence suggested that the stem cell properties render ATLL cells resistant to therapy and that the clinical effect of IFN-α is associated with inhibition of the stem cell properties, including the SP phenotype.

Materials and methods

Cell culture. The human ATLL cell lines (ATL-2, ATL-16T, ATL-35T, ATL-43Tb, Sez627C, SezM3, and ED40515) were kindly provided by Dr. Michiyuki Maeda (Institute for Virus Research, Kyoto University) [18]. MT-1, MT-2, MT-4, and SLB-1 were kindly provided by Dr. Mitsuaki Yoshida (Institute of Medical Science). HUT102 was obtained from ATCC (Manassas, VA, USA). Cells were cultured in RPMI1640 (Wako, Osaka, Japan) containing 10% fetal bovine serum (FBS), 50 U/mL penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine. For non-serum culture, ALyS505N-0 (without IL-2) medium (Cell Science & Technology Institute, Miyagi, Japan) was used.

Antibodies. Monoclonal antibodies that were unlabeled or conjugated with phycoerythrin (PE), fluorescein isothiocyanate (FITC), or allophycocyanin (APC) were obtained from companies described as follows. Anti-CD2, CD3, CD5: Beckman Coulter (Fullerton, CA, USA); CD48, CD49d, CD49e, TCRV beta2: Immunotech (Marseille, Cedex, France); CD52: Serotech (Birmingham, AL, USA); CD133: Miltenyi Biotech (Bergisch Gladbach, Germany); CD155: Lab Vision (Fremont, CA, USA); CD277: eBioscience (San Diego, CA, USA); CD1a, CD1b, CD1d, CD4, CD6, CD7, CD8, CD9, CD10, CD11a, CD14, CD19, CD20, CD25, CD26, CD27, CD28, CD31, CD34, CD38, CD40, CD40L, CD43, CD44, CD45RA, CD45RO, CD47, CD49f, CD54, CD56, CD60, CD69, CD80, CD86, CD90, CD95, CD99, CD106, CD110, CD117, CD121a, CD123, CD124, CD127, CD135, CD150, CD155, CD165, CD195, CD200, CD220, CD243, CD318, CD338; HLA-DR: BD PharMingen (San Jose, CA, USA). CD29 and CD82 were produced in our laboratory. Anti-STAT1, STAT3, STAT5, STAT6, phospho-STAT1 (pY701), phospho-STAT3 (pY705), phospho-STAT5 (pY694), phospho-STAT6 (pY641): BD Pharmingen. Anti-phospho-ERK (pT202/pY204): Cell Signaling Technology (Danvers, MA, USA). Anti-phospho-p38 (pT180/pY182), phospho-JNK (pT183/pY185): New England Biolabs (Ipswich, MA, USA).

Flow cytometry and SP analysis. To identify SP cells, the cells were washed and suspended in HBSS medium (Invitrogen, Carlsbad, CA, USA) containing 2% FBS, 10 mM Hepes buffer, and antibodies (HBSS+ medium). The samples were then incubated with 5 mM Hoechst 33342 dye (Sigma–Aldrich, St. Louis, MO, USA) at 37 °C for 60 min with or without 0.5 mM verapamil (Sigma–Aldrich), which is an inhibitor of some ABC transporters. The cells were counterstained with 2 μg/ml propidium iodide (PI) (Sigma–Aldrich) to label dead cells and were analyzed using a FACSVantage SE cell sorter (Becton–Dickinson, Mountain View, CA, USA) by a dual-wavelength analysis (blue, 424–444 nm; red, 675 nm) after excitation with 350-nm UV light. For SP-sorting and culture assays, the cells were gated to exclude PI+ cells and sorted from SP and non-SP (MP) fractions.

For phenotypic analysis of the cell lines, the cells were also stained with the above monoclonal antibodies for 1 h at 4 °C. When unlabeled antibodies were used, FITC-conjugated goat anti-mouse IgG antibody was used as a secondary antibody after incubation of the primary antibodies. Finally, the cells were washed and resuspended in HBSS+ medium containing PI, then analyzed on a FACSVantage SE or FACSAria (Becton–Dickinson).

Treatment with IFNs. IFNs (α, β, and γ) were obtained from Prospec-Tany TechnoGene (Einstein, Rehovot, Israel) and PeproTech EC (London, UK). For treatment with IFNs, ATL-43Tb cell line was cultured in non-serum medium at 1×10^5 cells/ml. IFNs were added at concentrations ranging from 0.1 to 10 ng/ml, then cell cultures were done for an additional 30 min or up to 4 days (time points of 0, 2, 3, 4 days), followed by FACS analysis and Western blotting.

Western blot analysis. Following IFN treatment, cells were collected and suspended in lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP40, 1 mM pyrophosphate sodium salt, 10 mM NaF, and 2 mM vanadate, protease inhibitor cocktail). After centrifugation, the supernatant was suspended and boiled in SDS–PAGE sample buffer, then loaded 20 μg/lane in 10% SDS–PAGE gel. The detection was carried out using ECL system (GE Healthcare, Little Chalfront Buckinghamshire, UK) according to the manufacturer's instruction.

Results

Identification of SP cells in ATLL cell lines

As ATLL is a highly chemoresistant neoplasm, we speculated that ATLL cells contain SP cells, which often

express MDR1 or ABC transporters genes. We performed SP analysis of a total of nine cell lines including ATLL-derived cell lines (HUT-102, SezM3, ATL-35T, Sez627C, ED-40515, and MT-1) and HTLV-I-transformed cell lines (SLB-1, MT-2, and MT-4). Among these cell lines, HUT102 contained a minor population of SP cells (0.47%), but others did not contain detectable SP cell populations (data not shown).

We further analyzed other ATLL-derived cell lines (ATL-2, ATL-16T, and ATL-43Tb) and found that ATL-2 (5.5%), ATL-16T (0.93%), and ATL-43Tb (3.0%) contained a higher level of SP cells (Fig. 1A). In each case, the SP cell populations were decreased greatly in the presence of verapamil, suggesting that these cells were indeed bona fide SP cells [4,6,9]. Our data therefore indicated that some ATLL-derived cell lines contained significant number of SP cells.

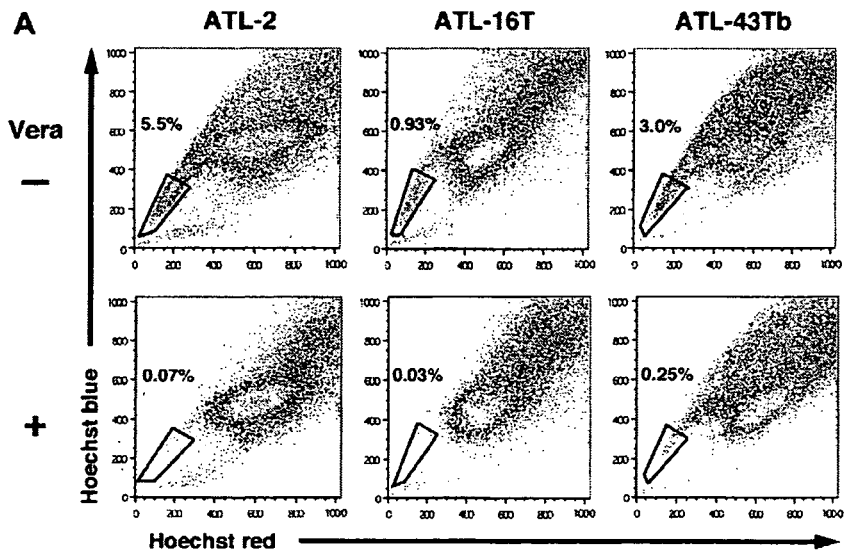
Expression of HSC-related markers on ATLL cell lines

We next carried out extensive cell marker analysis to identify a minor subpopulation having stem cell properties in ATL-2, ATL-16T, and ATL-43Tb cell lines, analogous to the strategy used to identify CSC of other tumors [1]. A total of 67 cell surface markers, including T-cell markers and stem cell markers, were analyzed (see Materials and methods). Human HSC are $CD34^+/CD38^-$ and $CD90^+/CD34^+$ cells are also shown to have stem cell properties [17]. In addition, recent studies indicate that CD48 is a member of the SLAM (CD150) family and is important for self-renewal in HSC [16]. Interestingly, several HSC-related markers, such as CD48 (all cell lines), CD90 (ATL-16T), CD123 (IL3-Rα, ATL-16T), CD150 (ATL-2, ATL-16T), and CD338 (ABCG2, all cell lines), were expressed. These findings suggest that ATLL cells recaptured cell surface characteristics of stem cell phenotype.

We subsequently focused on the HSC-related markers (CD34, CD38, CD48, and CD90) and characterized their expression on each cell line (Fig. 1B). While all cell lines tested were $CD34^-/CD38^-$ and CD48 positive, 5 of 12 were CD90 positive. Therefore, CD48 and CD90 are candidates for surface markers that may be associated with stem cell properties in many cases of ATLL. However, we could not detect a typical small subpopulation of so-called "cancer stem cells" by cell surface antigen analysis.

Repopulation of the original cell line pattern by both SP and non-SP cells

We compared the ability of SP and non-SP cells of the above ATLL cell lines to produce SP cells, expecting that since CSC are contained only in the SP fraction but not the non-SP fraction, we can prospectively isolate the CSC from ATLL cells. We stained each cell line with Hoechst 33342, and sorted them into the SP and non-SP (main population, MP) fractions by flow cytometry (Fig. 2A). The SP and MP-derived cells were then separately expanded and cultured for an additional 8–14 days. Unexpectedly in each cell line, we found that both SP and MP-derived cells could repopulate the SP fraction to reconstitute the original cell line pattern. Similar result was reported recently in C6 glioma cell line [10], which conflicted with a previous study [6]. These results therefore indicated that both SP and non-SP cells have the same potential to generate SP cells within the clone, suggesting that the SP phenotype is not identical with the stem cell population in ATLL.



B

	SP	CD48	CD90
ATL-2	+	++	-
ATL-16T	+	+/++	+/++
ATL-43Tb	+	+	-
ATL-35T	-	+	-
ED40515	-	+	-
Hut102	+	-/+	+
Sez627c	-	++	-
SezM3	-	++	++
SLB-1	-	++	++
MT-1	-	++	-
MT-2	-	-/+	++
MT-4	-	++	-

Fig. 1. (A) Analysis of SP cells in ATLL cell lines. The SP regions are indicated by a trapezoid on each panel. ATL-2, ATL-16T, and ATL-43Tb contained abundant levels of SP cells (upper panels), which were reduced by treatment with verapamil (lower panels). Vera, verapamil. (B) Summary of SP phenotype and expression of CD48 and CD90 in the ATLL cell lines. A broad range of expression was indicated by the symbols of −/+ and +/++.

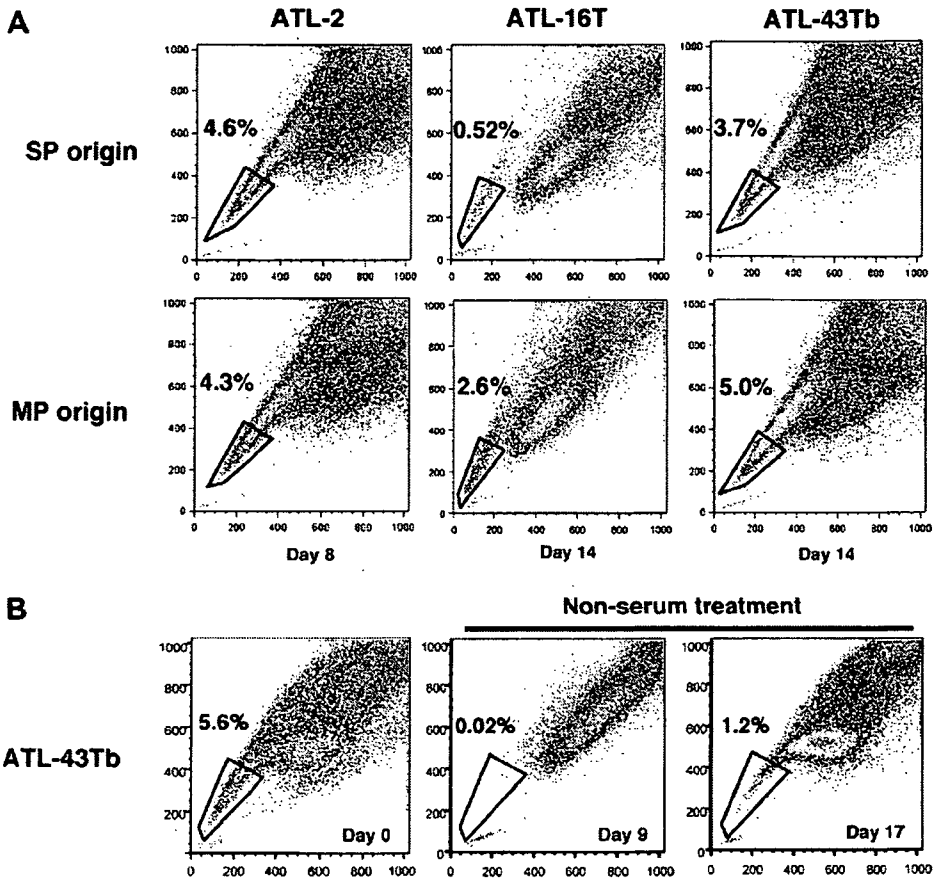


Fig. 2. (A) Cell sorting and culture assay for SP cells. Following cell sorting by FACS, SP, and MP were cultured and evaluated for the appearance of SP cells. For each cell line, culturing of both SP and MP cells led to the regeneration of SP cells. (B) Serum-depletion of ATL-43Tb. Following cell culturing, SP cells disappeared once within 9 days, and then repopulated within 17 days.

Non-serum treatment of ATLL cell lines

Although SP phenotype and HSC-marker expressions were observed in ATLL cells, a typical small subpopulation of CSC was not identified. However, it is our hypothesis that stem cell characteristics play an important role in this difficult-to-treat disease. As the SP phenotype has significant correlation with chemoresistance associated with the expression of ABC transporter genes, we next attempted to identify factors that affect the proportion of SP fraction. Since serum contains endogeneous factors that may interfere with our pharmacological assays, we first compared the fate of SP cells cultured in serum-containing medium versus serum-free medium. SP cells in ATL-2 were not affected by serum depletion whereas SP cells in ATL-16T disappeared within 9 days and did not reappear within 31 days (data not shown). On the contrary, SP cells of ATL-43Tb disappeared once within 9 days but reappeared within 17 days (Fig. 2B). Then they recovered to the same level seen with serum-containing culture with increasing passage numbers, so we used ATL-43Tb for the rest of assays.

Reduction of SP cells by IFN-α

IFN-α is sometimes used successfully for treatment of ATLL following failure of conventional therapy. Therefore, we speculated that IFN-α interferes with the stem cell properties of ATLL, which are the putative reason for resistance to therapy. We treated ATL-43Tb cells cultured in serum-depleted medium with IFN-α, -β, and -γ. After 3 days of culture, we discovered that only IFN-α significantly reduced the proportion of SP (Fig. 3A). No obvious cell death was observed during the experiment. Our following experiments were then designed to determine the optimal dose and schedule of IFN-α for SP reduction. SP was increasingly reduced with increasing concentrations of IFN-α at day 3 of treatment, with the effective concentrations being 1–10 ng/ml (Fig. 3B). We also found that the effectiveness of treatment with IFN-α correlated with duration of culture, reaching a maximum effect at day 4 (Fig. 3C) and this effect required continuous existence of IFN-α in the medium (data not shown).

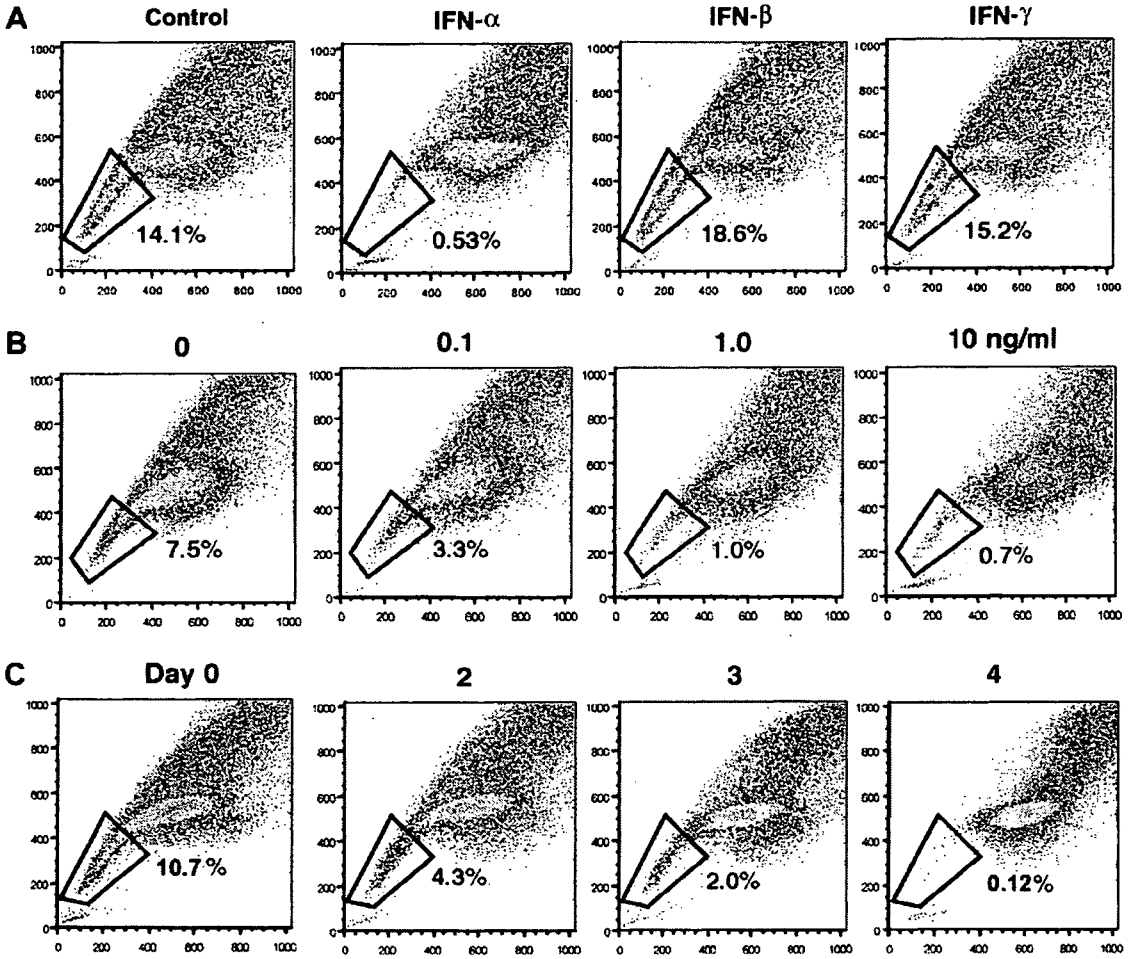


Fig. 3. Effect of IFNs on SP cells. (A) ATL-43Tb cells in serum-depleted medium were treated with IFN-α, -β, and -γ at a concentration of 10 ng/ml. After 3 days of culture, only IFN-α significantly reduced the proportion of SP cells. (B) Enhanced concentrations of IFN-α resulted in increasing reduction in SP levels, with the effective concentrations being 1–10 ng/ml at day 3. (C) Time course analysis of IFN-α effect. Increasing reduction of SP populations was seen with increasing duration of incubation, with the maximum effect seen on day 4.

IFN-α-mediated increase in CD25-positive cells

Since IFN-α can have an effect on multiple cellular functions, reduction of SP by IFN-α may correlate with cell differentiation and intracellular signaling events in the malignant clone. Normal T-lymphocytes are activated by IFN-α to upregulate interleukin-2 receptor α (IL-2Rα, CD25), and are also sensitized to IL-2-induced proliferation [19,20]. We thus evaluated potential IFN-α-mediated alterations in cell surface antigen expressions on ATL-43Tb cells. Following IFN-α treatment, the cells were stained with a panel of antibodies for selected T-cell and HSC markers (CD2, CD3, CD4, CD8, CD24, CD25, CD34, CD44, CD45RA, CD45RO, CD95, and CD117 (c-kit)), with only the level of CD25-positive cells being significantly increased (form 55% to 98% at 4 days) (Fig. 4A). Since CD25 is a marker of activated T-cells, the most differentiated form of T-lymphocyte, IFN-α may promote the differentiation of the immature ATLL cells into a mature type, associated with a concomitant increase in drug sensitivity.

IFN-α-mediated phosphorylation of STAT1, STAT5, and ERK

We next examined changes in intracellular signaling pathways following treatment with IFN-α. Since IFN-α enhances tyrosine phosphorylation of STAT family in normal T-cells [20], reduction of SP should be correlated with JAK/STAT pathway even in the malignant-transformed T cells. The ATL-43Tb cells were treated with IFN-α, -β, or -γ, then phosphorylation of STAT1, STAT3, STAT5, and STAT6 was analyzed by Western blotting (Fig. 4B). In 30 min, STAT1 and STAT5 were significantly phosphorylated by IFN-α and -β, while others were only weakly phosphorylated. Continued culturing of the cells for an extended period of time showed that only IFN-α induced the continuous phosphorylation of STAT1 and STAT5

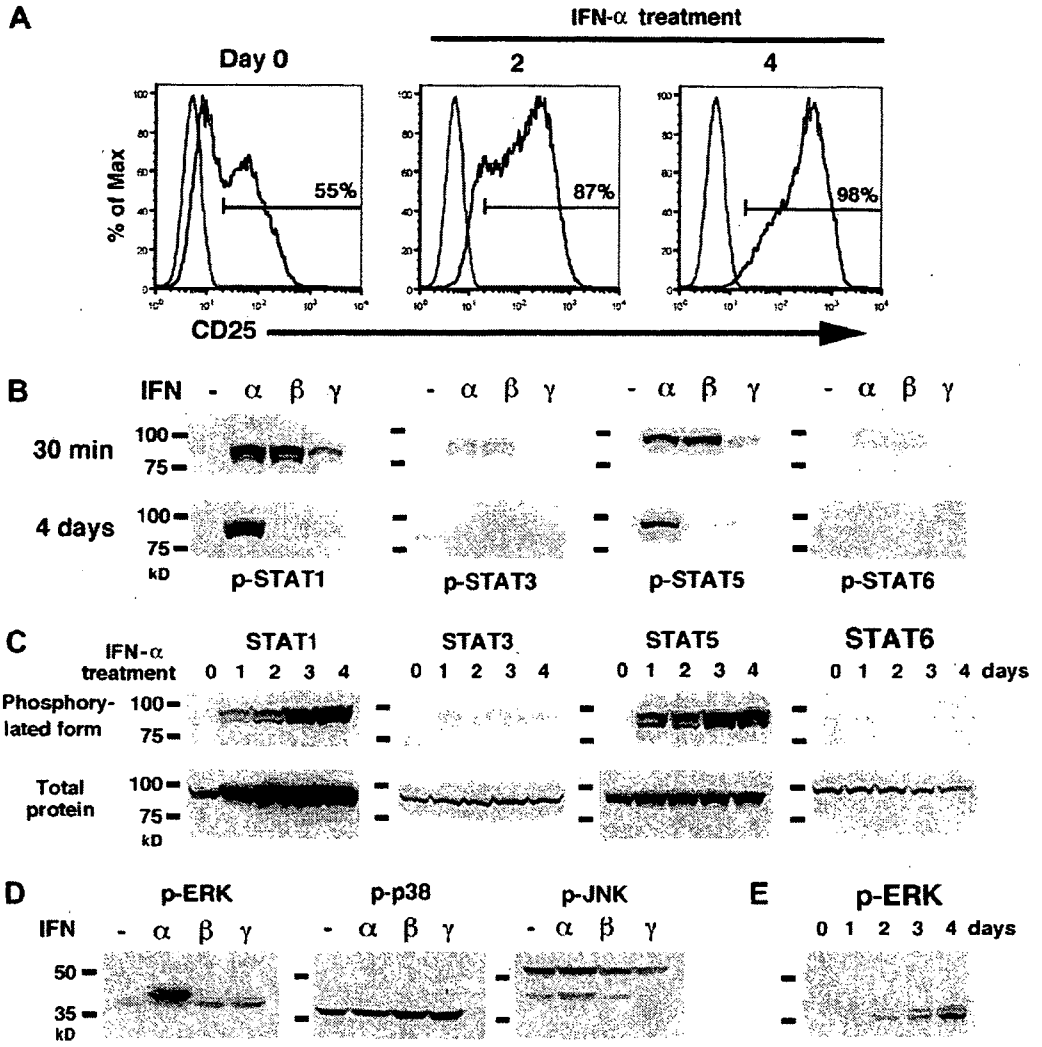


Fig. 4. (A) Effect of IFN-α on cell surface markers. Following IFN-α treatment, ATL-43Tb cells were stained with antibodies against selected surface antigens at several time points. Only CD25 was found to be significantly upregulated as shown in each histogram. Left peak in each plot, control staining without antibody. Right peak, CD25 staining. (B) Effect of IFN-α on JAK/STAT pathways. After treatment with IFNs, phosphorylated forms of STAT1/3/5/6 were detected by Western blotting. In 30 min, STAT1 and STAT5 were significantly phosphorylated by IFN-α and -β (upper panels), but others were weakly phosphorylated. At day 4, only IFN-α induced the phosphorylation of STAT1 and STAT5 (lower panels). (C) Time course analysis of STAT phosphorylation. STAT1 and STAT5 phosphorylation gradually increased from day 1–4. Upper panels, blotting of phosphorylated STATs. Lower panels, total protein of STATs. (D) Effect of IFN-α on MAP kinase pathway. Only ERK was phosphorylated. (E) Time course analysis of ERK phosphorylation.

following 4 days of treatment. Time course studies of STATs phosphorylation demonstrated that phosphorylation of STAT1 and STAT5 gradually increased over the 4 days culture period (Fig. 4C). These results hence demonstrated that the response to IFN-α by the JAK/STAT signaling pathway was conserved in ATLL cells.

We carried out similar assays to evaluate the effect of IFN-α on effectors of the MAP kinase signaling pathway, including ERK, p38, and JNK. Correlation between IFN-α and MAP kinase pathway has also been demonstrated in lymphocytes and leukemia cells [21,22]. Among these proteins, only ERK was phosphorylated by IFN-α, with a gradual increase in phosphorylation level being observed (Fig. 4D and E). Although this response was quite different from that seen in other type of cells, ERK may be a key molecule in the signal transduction of ATLL cells.

Discussion

We did not detect a typical small subpopulation of CSC in the ATLL cell lines tested, however, we did identify SP cells in the four ATLL cell lines. In contrast to previous reports, SP and non-SP cells could both reconstitute the original cell line pattern, suggesting that the SP phenotype is not a CSC marker in ATLL. We also performed extensive surface marker analyses and found that many HSC-related markers, including CD48 and CD90, were present on the ATLL cell lines. Although these two antigens are not normally expressed on mature T-cell surface, our data

suggest that ATLL cells reacquired stem cell-like properties while concurrently re-expressing these markers.

On the other hand, we discovered that IFN-α reduced the SP cells without decreased cell viability. The combination of IFN-α and other agents has been reported to exhibit clinical effectiveness in some patients who had failed initial therapy. As the SP phenotype is generated by ABC transporter or MDR genes, IFN-α may inhibit the activities of these gene products, resulting in the observed clinical effect. We also demonstrated that STAT1/5 and ERK were phosphorylated by IFN-α during this process. These observations indicate that the JAK/STAT and MAP kinase pathways are important for the maintenance of stem cell properties, including the SP phenotype.

In conclusion, our results suggest that the reduction of SP by IFN-α is correlated directly or indirectly with alterations of key cellular signaling pathways, changes which may be linked to the clinical effectiveness of IFN therapy in patients with chemoresistant ATLL. Further understanding of the stem cell properties of ATLL may therefore be a key to the development of new therapeutic approaches for this disease.

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