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.

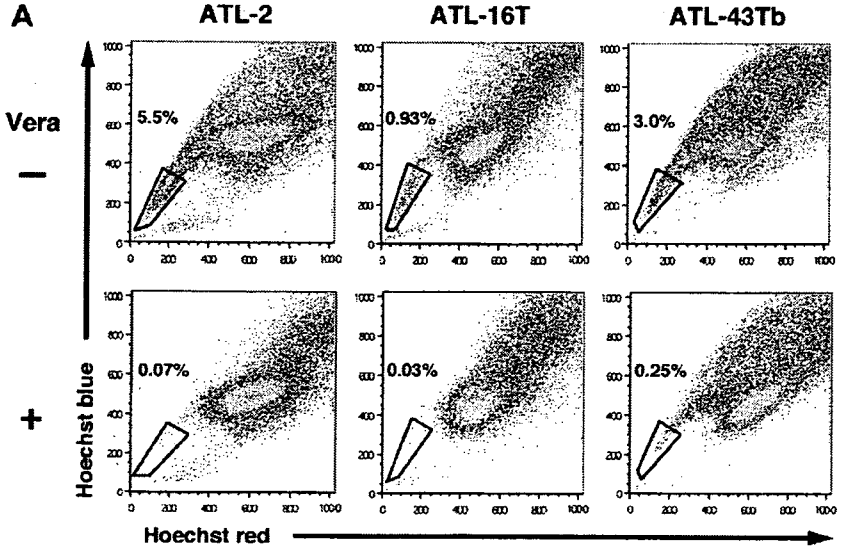
Expression of HSC-related markers on ATLL cell lines

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 +/++.

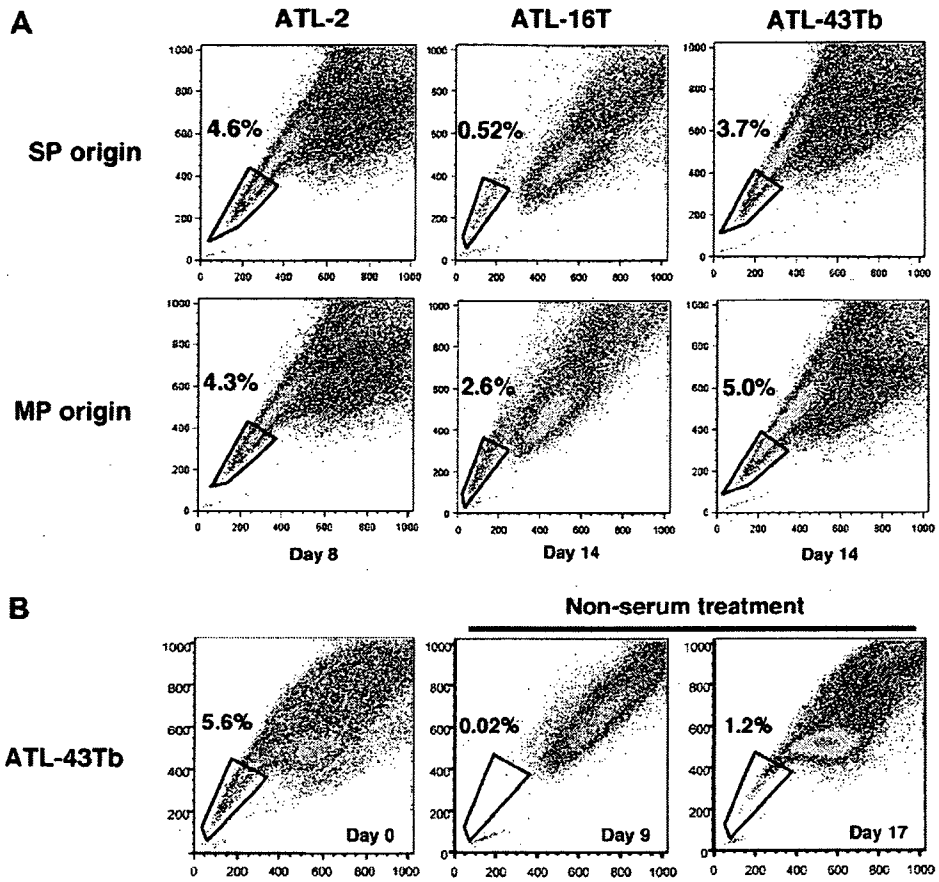


Fig. 2. (A) Cell sorting and culture assay for SP cells. Following cell sorting by FACS, SP, and MP were cultured and evaluated for the appearance of SP cells. For each cell line, culturing of both SP and MP cells led to the regeneration of SP cells. (B) Serum-depletion of ATL-43Tb. Following cell culturing,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

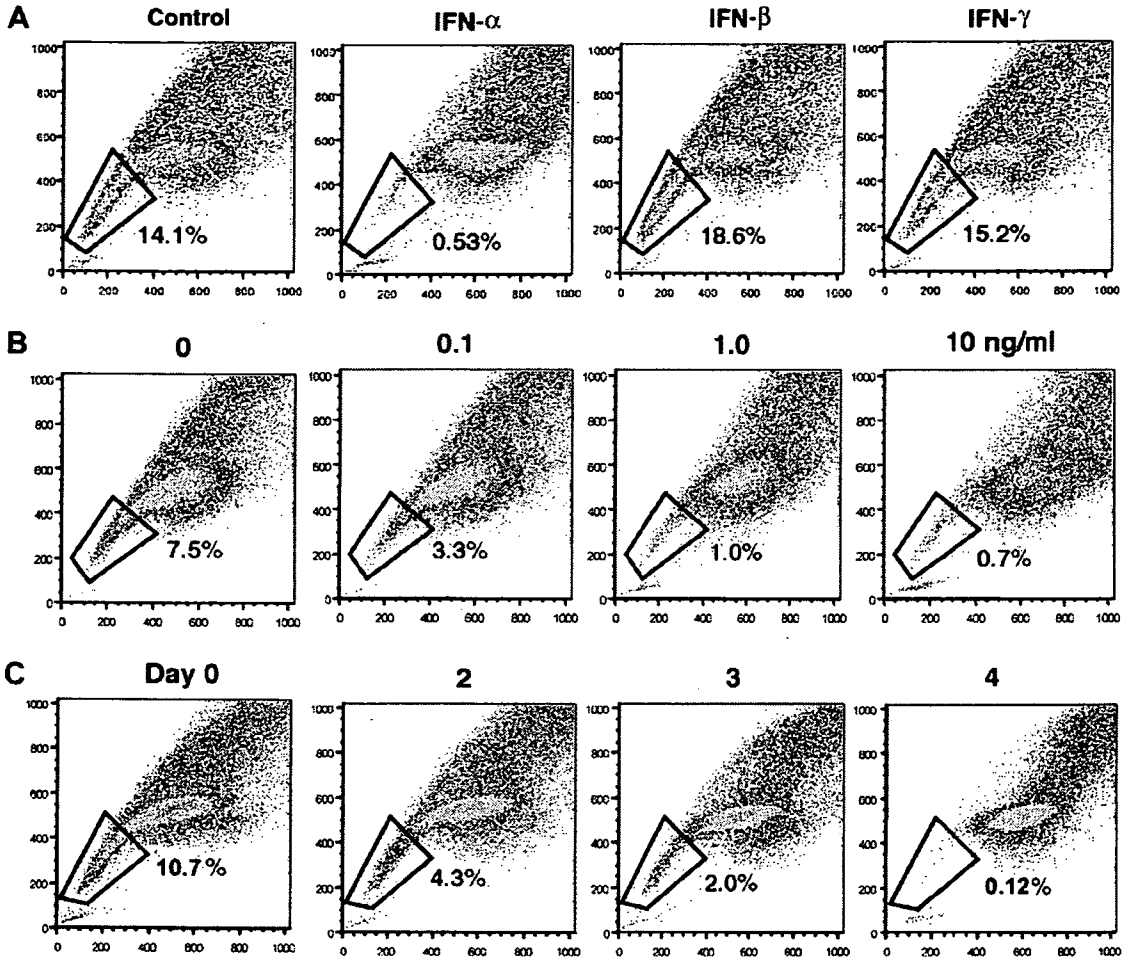


Fig. 3. Effect of IFNs on SP cells. (A) ATL-43Tb cells in serum-depleted medium were treated with IFN-α, -β, and -γ at a concentration of 10 ng/ml. After 3 days of culture, only IFN-α significantly reduced the proportion of SP cells. (B) Enhanced concentrations of IFN-α resulted in increasing reduction 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

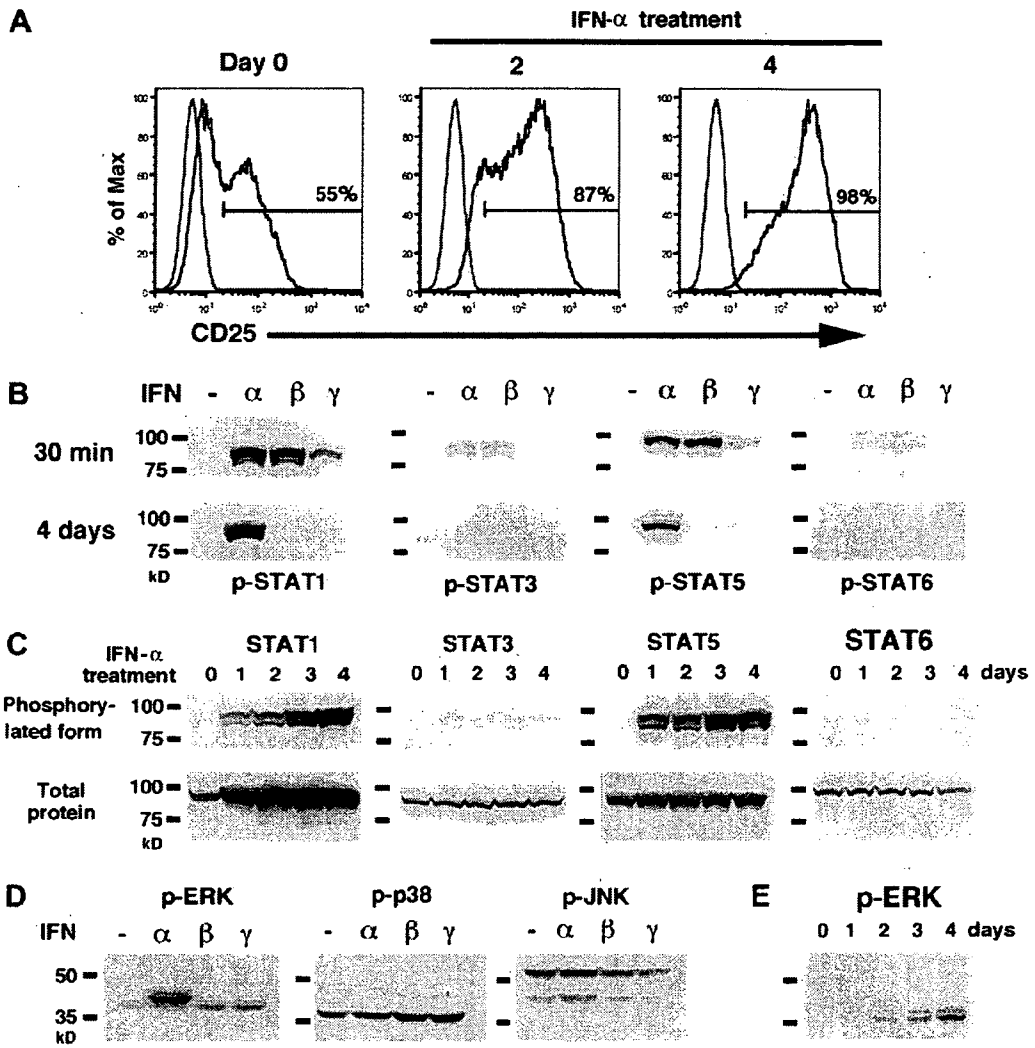


Fig. 4. (A) Effect of IFN-α on cell surface markers. Following IFN-α treatment, ATL-43Tb cells were stained with antibodies against selected surface antigens at several time points. Only CD25 was found to be significantly upregulated as shown in each histogram. Left peak in each plot, control staining 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.

Acknowledgments

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Could Serum Antibody to Poly(ADP-Ribose) and/or Histone H1 Be Marker for Senile Dementia of Alzheimer Type?

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ABSTRACT: **Poly(ADP-ribosyl)ation has been focused on ischemic injury in the brain in relation to Alzheimer's disease (AD). We have measured IgG antibodies against poly adenosine diphosphate-ribose (pADPR) as well as histone H1 (H1) in 26 patients with either AD or with senile dementia of Alzheimer type (SDAT), and found that 80.7% (21/26) were positive for anti-pADPR IgG antibodies. Anti-H1 IgG antibodies were less positive (57.6%) (15/26) than anti-pADPR IgG antibodies, however, titers of both antibodies were well correlated ($r = 0.768$). Meanwhile, similar studies on 32 patients with systemic lupus erythematosus (SLE) who were positive for anti-pADPR antibody showed poor correlation ($r = 0.184$) and the difference in the correlation was statistically significant ($r < 0.01$). It is worthy of remark that anti-double-stranded (ds) DNA antibody, which is the hallmark of SLE, was negative in all dementia patients. Together with the findings that major subclass in dementia is both IgG1 and IgG2 and that in SLE was IgG2, the mode of production of anti-pADPR antibody in AD and SDAT is under different regulation mechanisms from that in SLE. Given the evidence that major target for ADP-ribosylation is H1 molecule, the association between anti-pADPR and anti-H1 in AD/SDAT makes sense and supports the concept that modification of proteins renders them immunogenic. Whatever the regulation is, parallel assay of two antibodies above would be of use not only for monitoring the disease process but also as a prodrome for possible subsets of SDAT and AD.**

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KEYWORDS: **poly(ADP-ribose); ADP-ribosylation; antipoly (ADP-ribose); Alzheimer's disease; blood–brain barrier (BBB); autoantigen**

INTRODUCTION

Recent reports on patients with Alzheimer's disease (AD) that had a shift of histone H1 (H1) to the cell surfaces from nuclei in the astrocytes[1] as well as high levels of adenosine diphosphate (ADP) ribosylation reaction in the atrocytes[2] reminded us of our previous finding that immunization of rabbits with ADP-ribosylated histone enhanced the immune response not only to poly(ADP-ribose) (pADPR) but also to histone itself.[3] The presence of anti-pADPR antibody in patients with systemic lupus erythematosus (SLE) was also reported first by Kanai *et al.*[4] Dimerization of H1 by means of ADP-ribosylation reaction[5] is theoretically of value in the induction of autoimmunity in view of an established system of hapten ADP-ribose and carrier H1. Based on these findings, we measured the IgG immune responses to both H1 and pADPR in 20 patients with senile dementia of Alzheimer type (SDAT) and in 6 patients with AD; none had rheumatic diseases. Nevertheless, we have found that these patients respond to both antigens. The tight metabolic relationship between AD and pADPR and/or histone,[1,2] and immunological interrelationship between pADPR and histone[3] prompted us to study and compare the mode of immune responses to these antigens between patients with AD or SDAT, which are not affirmed as yet to be autoimmune diseases and those with SLE, a representative systemic autoimmune disease that shows high immune responses to nuclear antigens including histones and pADPR.[4,6] In these studies, a number of patients with AD and SDAT were small. Instead, we included serum samples from 59 elderly without AD or SDAT.

MATERIALS AND METHODS

pADPR was synthesized from calf thymus nuclei in the presence of nicotinamide adenine dinucleotide and was purified as previously reported.[3] H1 was separated by cationic ion exchange chromatography (HiTrapSP; Amersham Biosciences, Uppsala, Sweden) from total histones purified from nuclei of HL-60 cells as previously reported.[7] Double-stranded (ds) DNA was isolated as the pure form of HL-60 cell nucleosomes, as described.[7] Purification of genomic DNA was done as well. pADPR thus purified was free from H1 and *vice versa*. Also, dsDNA was guaranteed to be free from nucleosomal proteins, as far as tested by agarose gel electrophoresis. Assay for IgG antibodies to each antigen was carried out by enzyme-linked immunosorbent assay (ELISA) in which antigens were directly coated to microtiter plates (Immulon 2HB, Chantilly, VA) under 25 mM Tris (pH 7.4) buffer containing

250 mM NaCl in order to eliminate the bias of precoating with glues, such as poly-L-lysine as described.[7] Diagnose of SDAT and AD were made according to DMS-IV (*Diagnostic and Statistical Manual of Mental Disorders*, 4th ed., American Psychiatric Association, Washington, D.C., 1994), and diagnosis for SLE was made according to *Updating the American College of Rheumatology Revised Criteria for the Classification of SLE (1997)*. Twenty-six patients with AD or SDAT who participated in this research were over 65 years old; whereas the age in average of 59 elderly participated as controls without particular diseases was 77 years. Moreover, the age in average of 40 patients with SLE was at least under 60 years. In order to obtain basal levels for anti-pADPR and anti-H1 antibodies, sera of 28 healthy persons without particular diseases under 60 years of age were used: their means plus 2 SD were adopted as a basal level of individual antibodies: the former was 0.173 A405 units and the latter was 0.105 A405 units. All sera collected from individual participants were stored at –70°C until use. It should be mentioned that this study was approved by the ethics committee of this hospital and bloods were drown under informed consent of subjects/patients or their guardians in cases of dementia patients.

RESULTS

Six (100%) of 6 AD patients and 15 (70%) of 20 SDAT patients were positive for anti-pADPR antibodies (overall positivity of anti-pADPR antibody for patients with SDAT and AD was 80.7%, and 32 (80%) of 40 patients with SLE were positive for anti-pADPR antibodies. Also, 15 (57.6%) of 26 patients with SDAT or AD were positive for anti-H1 antibodies, whereas 12 (30%) of 40 patients with SLE were positive for anti-H1 antibodies. Profiles of autoantibodies in the two diseases positive for anti-pADPR antibodies are shown in FIGURES 1 and 2. The level of anti-pADPR antibodies in patients with SLE (FIG. 2) was higher than that in dementia (SDAT/AD) (FIG. 1) as was expected, however, the difference in the correlation coefficient (r) between two antibodies in an individual disease was statistically significant ($P < 0.01$); namely, the correlation coefficient (r) of 21 patients with dementia who were positive for anti-pADPR antibody was 0.768 (FIG. 1), while that of 32 SLE patients who were positive for anti-pADPR antibody was 0.184 (FIG. 2). It should be mentioned here that anti-dsDNA antibody, a hallmark of SLE, was 90% positive in patients with SLE as was expected, but it was totally negative in all patients with dementia (data not shown). To substantiate the relationship, we have further studied 59 cases of age-comparable (77 years old in average) elderly without either dementia or SLE and found that 37% (22/59) were positive for anti-pADPR IgG antibody but only 3.3% (2/59) were positive for anti-H1 IgG antibodies, none of 22 elderly positive for anti-pADPR being associated with anti-H1 at all (TABLE 1). Again, positive cases of either were negative

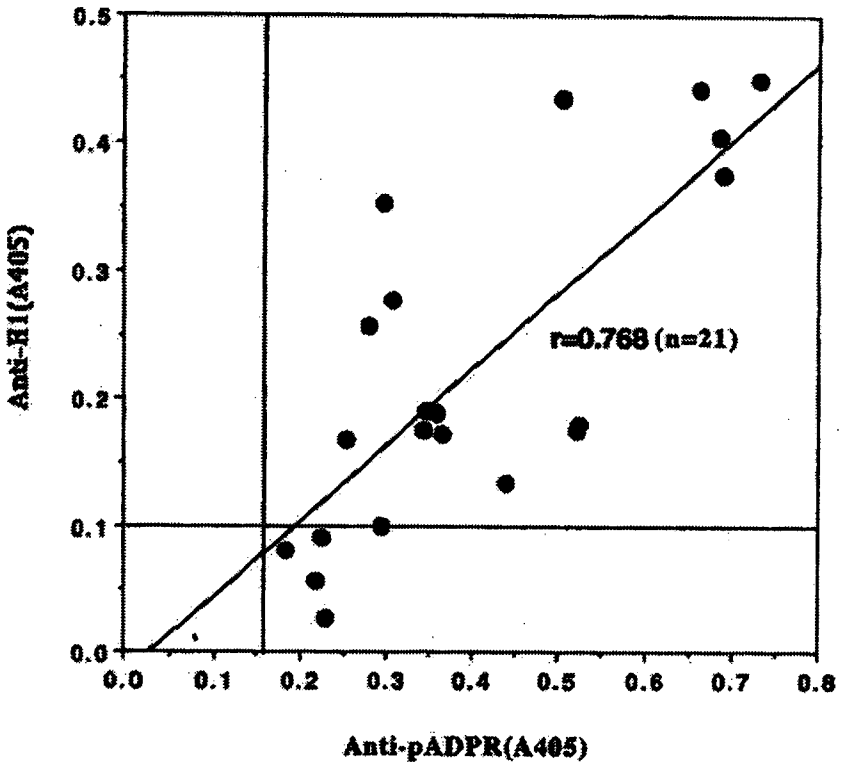

FIGURE 1. Correlogram between anti-pADPR antibodies and anti-H1 antibodies in 21 patients with SDAT and AD who showed positive for anti-pADPR antibodies determined by basal level (mean +2 SD) obtained from 28 healthy subjects with age under 60 years as described. Vertical line shows basal level of anti-pADPR (0.173 A405 units) and horizontal line shows that of anti-H1 antibodies (0.105 A405 units). Steep correlation curve in between basal lines is seen ($r = 0.768$).

for anti-dsDNA antibody (data not shown). Whether or not elderly positive for anti-pADPR antibody are prodrome for AD or SDAT is of great concern, therefore we must study carefully the outcome of anti-pADPR antibody-positive elderly.

DISCUSSION

Given the historical background on pADPR mentioned above, data presented here strongly suggest that H1 is ADP-ribosylated in dementia but not in SLE except for two patients (5%) with high anti-H1 titers (>0.5 A405 units) shown in FIGURE 2. Moreover, the possible presence of ADP-ribosylated H1 on the surface of astrocytes is accessible to the immune system through the

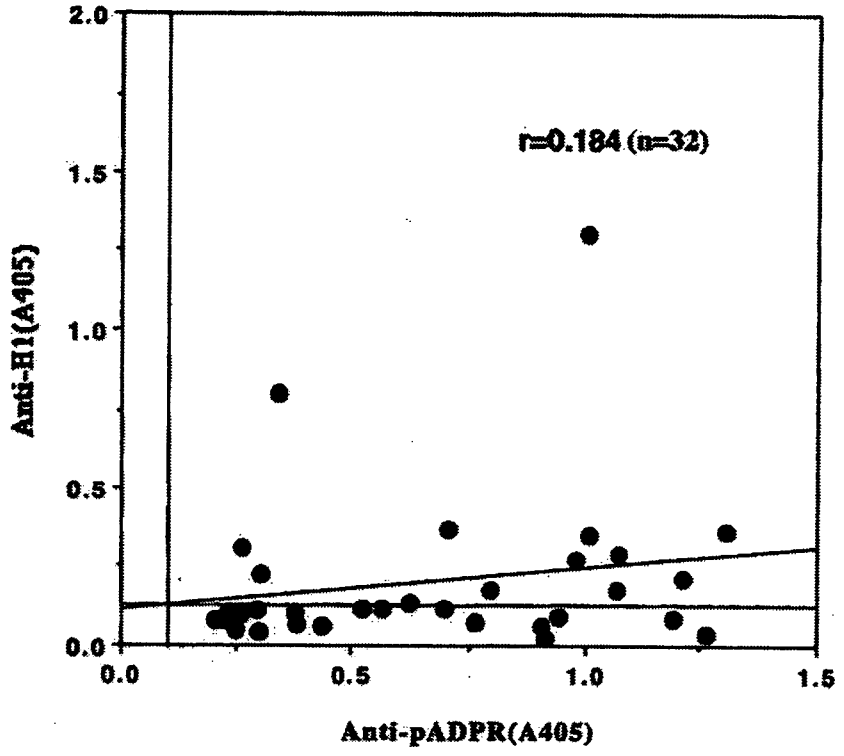

FIGURE 2. Correlogram between anti-pADPR antibodies and H1 antibodies in 32 SLE patients positive for anti-pADPR antibodies: basal levels for both antibodies are the same as in FIGURE 1. However, in contrast to cases of SDAT and AD, correlation curve was shallow, indicating low coefficient ($r = 0.187$).

hampered blood–brain barrier (BBB) that is probable in neurodegenerative disorders.[8,9] The difference in antibody subclass between SDAT or AD and SLE, namely the former two cases were IgG1 and IgG2 and the latter was IgG2 alone (data not shown), suggests that the production of autoantibodies occurs under different regulation mechanisms in two diseases. To our understanding, autoantibodies against nuclear antigen-associated autoantigens in degenerative brain diseases have not been specially focused in the field of autoimmunity as well as rheumatic diseases. However, during search for the relationship between neuronal degeneration and autoimmunity in terms of deregulation of BBB, we have found several reports describing the autoimmune responses against not only nuclear chromatins, such as DNA and histones,[10] but also brain-associated antigens.[11,12] It is of note that D'Andrea argues that AD should be listed as an autoimmune disease.[13] Aside from whether or not SDAT and AD fulfill a criterion of autoimmune disease in the critical meaning, it could be excluded that the cases of AD and SDAT presented here are complicated with SLE.

TABLE 1. Immune responses to chromatins in 59 elderly (77 ± 7.7 years old) without dementia or autoimmune diseases

Occurrence of antichromatin antibodies				
H1	nucleosomal DNA	Genomic DNA	Nucleosome	poly(ADP-ribose)
2/59(3)	4/59(7)	4/59(7)	2/59(3)	22/59(37)

Parenthesis indicates % positive.

because it seems too high that 80.7% of AD and SDAT patients negative for anti-dsDNA IgG antibody are complicated with SLE or other collagen diseases. Whatever AD or SDAT is an additional autoimmune disease, a parallel assay for IgG autoantibodies against pADPR and H1 would be of use not only as a prodrome of patients with dementia (SDAT/AD) but also for monitoring the disease progress. Moreover, subgrouping of patients with AD and/or SDAT may also be possible according to the presence or absence of both antibodies to pADPR and H1 or single presence of either. In this respect, the findings that 19.3%(5/26) of AD and SDAT patients are negative for anti-pADPR antibodies and that 38.4%(10/26) of these patients are negative for anti-H1 antibodies should be taken into account. Whether or not elderly positive for anti-pADPR antibody mentioned above are a prodrome for AD or SDAT is of great concern, therefore we must carefully examine the outcome of them positive for anti-pADPR antibody. In cases of neuroleptic therapy, such as the phenothiazines to neurodegenerative disease, there happens the occurrence of anti-nuclear antibodies,[14] and, in fact, multicase families with Schizophrenia had anti-dsDNA antibodies,[15] but, we have not used such therapy in patients with AD/SDAT. In conclusion, as Meli *et al.*[16] described, it is quite reasonable that pADPR and/or pADPR polymerase (PARP) plays a key role in excitotoxicity and postischemic brain damage and that pADPR and/or PARP themselves serve as autoantigens as long as BBB is hampered, as proposed.[8,9]

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